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SECTION MEETINGS

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MINNESOTA	
University of Minnesota	March 10, 1948
PACIFIC COAST	
Permanente Foundation Hospital	February 25, 1948
SOUTHERN	
Louisiana State University	March 2, 1948

16322

Some Characteristics of the Anti-Estrous Factor in *Lithospermum*.*

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Two laboratories have independently reported that the herb *Lithospermum ruderale* when mixed with normal diet abolishes the estrous cycle in mice.[1,2] The present note describes further studies on modes of administration, site of action within the body, differential strain susceptibilities, distribution of the factor within the *Lithospermum* plant, as well as some preliminary fractionation work.

Experimental. 1. *Absorption from Pellets.* Daily vaginal smears, using the method previously described,[2] were made from large groups of Rockland Swiss strain virgin female mice between 2 and 4 months of age. Fifty of these mice showing least irregularity of estrus were selected. These were further segregated into 5 uniform weight groups. One lot was maintained as the control, each mouse receiving a subcutaneously implanted 50 mg beeswax pellet. Individuals of the second lot received similarly implanted pellets consisting of 25 mg of beeswax and 25 mg of the crude oil residue of the ether extraction of 100 g of powdered *Lithospermum*.† A neutral water extract of the same batch of *Lithospermum* was incorporated in similar proportions into other beeswax pellets, and implanted into a third group of mice. A fourth group received beeswax pellets con-

* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service. Technical assistants on the project were M. L. Drasher and Andrew Nowak.

1 Cranston, E. M., *J. Pharm. and Exp. Therap.*, 1945, **83**, 130.

2 Drasher, M. L., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 66.

† *Lithospermum* powder was prepared by milling the whole plant, including roots, stems, floral parts, stalks, and leaves. Florescent plants were collected in mid-June in the deserts of western Montana, and dried in the shade.

taining 2.5 mg of *iso*-androliclactone acetate.‡ A fifth group received beeswax pellets containing 10 mg of thiouracil.§ A daily estrous record for each of the mice comprising these groups had been kept for 20 days previous to pellet implantation; and vaginal smear estrous assay was continued for 40 days following the implantation. In only one of the 5 groups was there observable suppression of estrus, *i.e.*, the group receiving the water-soluble *Lithospermum* fraction.

The mean weights of each group of mice were taken weekly, starting with the time of pellet implantation and continuing during the experimental 40-day period. The weight curves were normal except for that of the thiouracil group, and to some extent that of the *iso*-androliclactone acetate group, which dropped somewhat initially, but recovered within 3 weeks. The drop in the thiouracil weight curve is taken to indicate that an effective anti-thyroid level of the compound was being maintained by absorption of the pellet, without exertion of any pronounced anti-estrous effect. It is not clear how thiouracil under these conditions would effect a weight loss; one may assume either some kind of side action, or assume that absorption from the pellet was so rapid as to constitute a gross overdose. Whether the *iso*-androliclactone acetate was similarly toxic was not clear, due to the questionable significance of its weight curve aberrancy.

2. *Strain Differences.* It was desirable to obtain additional data on strain differences in respect to susceptibility to the action of *Lithospermum*. The strains to be compared were the Rockland Swiss and the C_3H lines, the latter a high mammary tumor incidence strain. A set of 2 to 4 months old Rockland females was examined for estrous regularity, and all clearly irregular or persistently diestrous animals were discarded. The selected mice were divided into 2 lots, one fed stock Rockland diet, the other a 7% *Lithospermum* diet mixed with powdered stock Rockland diet, compounded into pellets. During the first week of feeding no vaginal smears were taken; thereafter for 2 months daily smears were made.

The estrus data for the second strain were obtained from females of a group of several hundred C_3H mice which had been placed on a *Lithospermum* diet shortly after weaning. Random samples from the control and experimental C_3H reservoirs were selected for 2-week assays when about 4 months of age. In a second instance, the C_3H females assayed were from a group which had been on a *Lithospermum* diet for about 8 months; and a third sampling was made of the C_3H stand-by group and their normal diet controls when the animals were about 14 months of age (*i.e.*, having been on the *Lithospermum* diet for about 12 months). All of the C_3H females, regardless of age, responded almost uniformly in showing a continuous anestrus, whereas only a partial inhibitory response characterized the Rockland females, confirming observations made earlier.[2] It is evident that the C_3H strain is considerably more susceptible to the anti-estrous factor in *Lithospermum* than is the Rockland strain. Also, it appears that the cyclic refractoriness described for the latter strain in our earlier paper[2] is largely absent in the C_3H strain.

Two groups of C_3H females, one having been on *Lithospermum* diet for 8 months, and the other on normal diet for a similar period of time, were assayed for estrus. The *Lithospermum* group had been sampled throughout the 8 months period, and the animals were found to have been in a state of continuous diestrus. The diet of these two groups was reversed: those which had been on *Lithospermum* were placed on normal diet, and conversely. The group which for the preceding 8 months had been in a state of continuous diestrus, immediately returned to relatively normal cyclicity; and the normal animals previously on the stock diet, responded within 96 hours to the *Litho-*

‡ *Iso*-androliclactone acetate was selected for test because it is thought to cause an accumulation of gonadotrophins in the anterior pituitary. The compound was supplied through the generosity of Dr. Gregory Pincus of the Worcester Foundation for Experimental Biology.

§ Supplied by Dr. R. O. Roblin, Jr., of the American Cyanamide Company, Stamford, Conn.

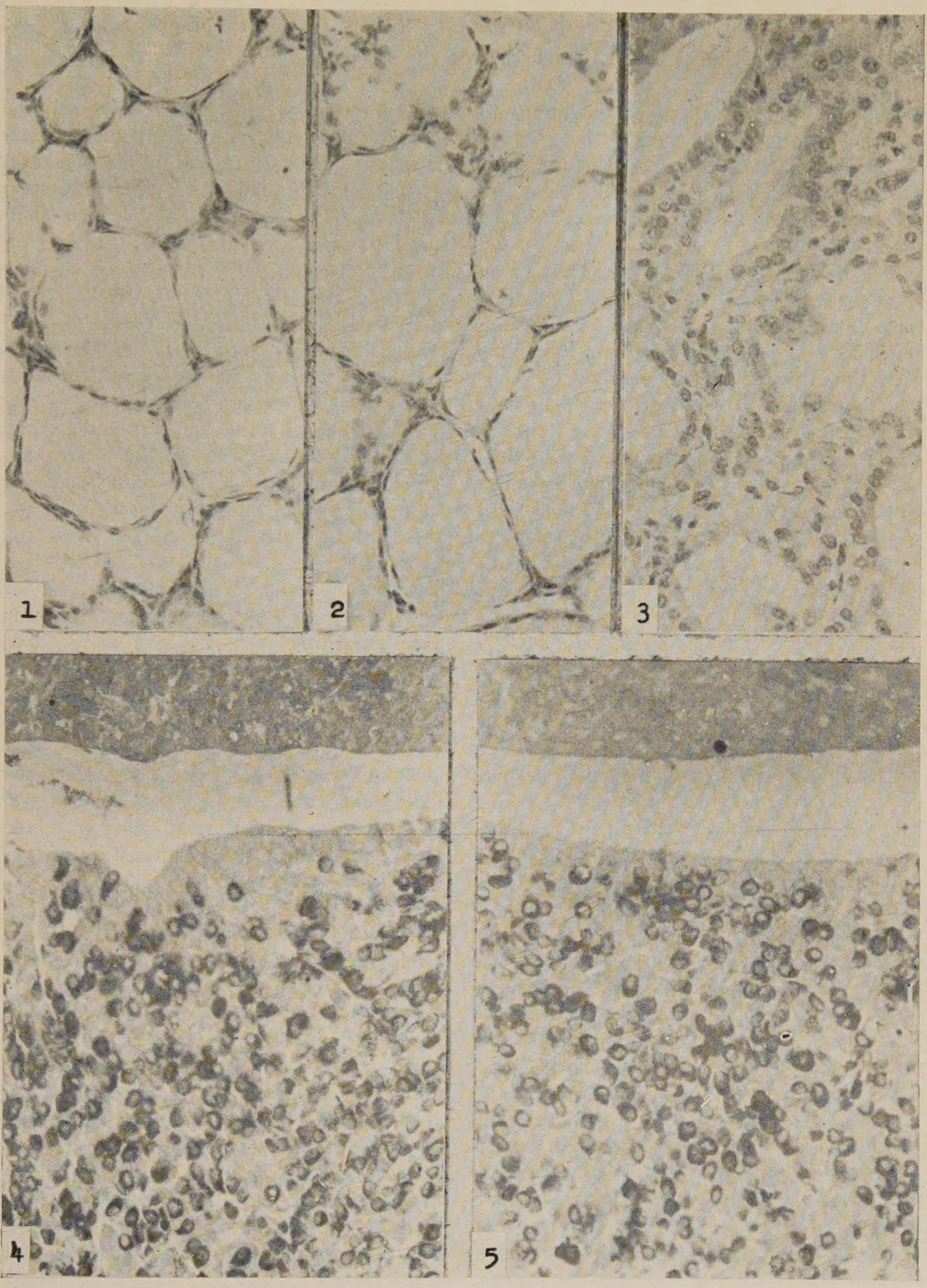

PLATE 1.

All photos taken under high-dry objective with × 12 ocular.

Figs. 1 and 4. Thyroid and anterior pituitary from 12-months-old C_3H female mouse which had been in continuous diestrus for 10 months, due to *Lithospermum* feeding.

Figs. 2 and 5. Thyroid and anterior pituitary from 12-months-old C_3H female mouse on normal diet.

Fig. 3. Thyroid from adult C_3H female mouse which had been on 0.1% thiouracil diet for 30 days.

spermum. The immediacy of the return to normal of the former group after being taken off the *Lithospermum* diet indicates that long exposure to the *Lithospermum* factor induces no permanent or irreversible changes within the body.

The weight history of female mice fed on *Lithospermum* diet from weaning, presents some points of interest. Such weight data were obtained each month beginning 5 months after the initiation of the experimental feeding. There was no significant weight difference between experimental and control groups. Cranston,[1] using larger doses—40% *Lithospermum* in the diet—reported that mice undergo an initial weight retardation from which they recover, however, in four months while still under treatment. Our weight data did not cover the age period between 2 and 7 months.

3. *Histology*. Studies were made of various tissues of C_3H mice which had been on 7% *Lithospermum* diet for from 10 to 12 months. No changes were observed in the thyroid epithelium and follicles in animals which for almost a year had been in sustained diestrus due to *Lithospermum* feeding (Fig. 1 and 2). The anterior pituitary, fixed and stained according to a method used earlier,[3] appeared to be normal in both control and experimental animals, no marked qualitative or quantitative changes being observed in any of the cell types (Fig. 4 and 5). The uteri appeared somewhat atrophic and were clearly in the anestrous condition. The ovaries were somewhat smaller than normal and showed some follicular atresia. This atresia was similar to but not as marked as that reported for *Lithospermum*-fed Rockland Swiss mice, whose ovaries underwent pronounced atresia following prolonged *Lithospermum* administration.[2] The mammary tissues of the experimental C_3H animals were rudimentary. It should be noted that control animals used for histological comparison with the experimental ones were sacrificed only when in true diestrus.

4. *The Thyroid*. The possibility that *Lithospermum* acts by suppression of the thyroid rather than by affecting the gonadotrophin-producing tissues of the pituitary, seemed to justify further investigation, despite the failure of the pellet-administered thiouracil to show any anti-estrous effect. Accordingly, two sets of mice were fed on diets consisting respectively of 0.1% and 0.5% thiouracil. These diets were fed to C_3H females with a history of normal estrous cyclicity. At these doses there was no disturbance of estrus. An observed body weight loss implies that enough thiouracil was absorbed to exert a systemic effect. Histologically the thyroids of mice so treated with thiouracil showed very marked hyperplasia (Fig. 3). These observations are consistent with the findings of Jones, Delfs, and Foote, who observed no change in the mating behavior of thiouracil-fed rats,[4] although gestation was often interfered with; but are at variance with those of Mann[5] who noted lengthening of inter-estrous periods in thiouracil-fed rats.

5. *Distribution of Factor within the Plant*. In all the preceding *Lithospermum* experiments, as well as in those of Cranston, the whole plant was used in the experimental diets. It was of interest to investigate the relative abundance of the anti-estrous factor in the various parts of the plant. Accordingly, dried *Lithospermum* plants were divided into (1) flowers and seeds, (2) roots, (3) leaves, (4) stems. These fractions were pulverized and made up to 15% with normal powdered Rockland mouse diet. Four groups of about 10 female Rockland mice each, on the stock diet, were assayed for estrous regularity for 10 days, then placed on the special diets. Daily estrous assay was continued during a 20-day experimental-diet period. The data are presented in Fig. 6, together with mean body weight curves. It is seen that the factor is practically absent from the stems, and maximally concentrated in the flowers and seeds. Return to normal diet resulted in almost immediate return to normal estrous cyclicity.

[3] Zahl, Paul A., *Z. f. Mikro-Anat. Forsch.*, 1937, **42**, 303.

[4] Jones, G. E. S., Delfs, E., and Foote, E. C., *Endocrinology*, 1946, **38**, 337.

[5] Mann, C. W., *J. Psychol.*, 1945, **20**, 91.

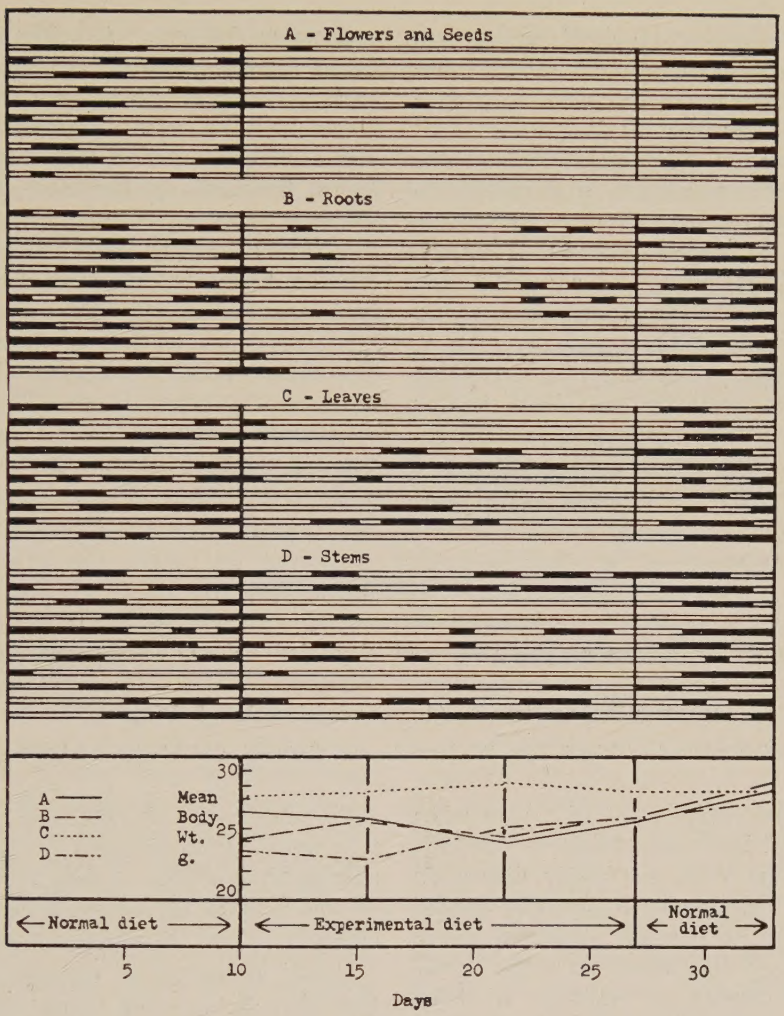

FIG. 6.

Estrous history of mice fed, respectively, on A—Flowers and Seeds; B—Roots; C—Leaves; D—Stems, of the plant *Lithospermum ruderale*. Pulverized, these materials were made up to 15% with normal powdered mouse diet, then compounded into pellets. The experimental feeding was preceded and followed by periods of normal diet feeding. The black horizontal areas indicate duration of estrus. The mean body weight curve for each group of mice is plotted below.

Discussion. It is clearly established that the *Lithospermum* factor, presumably in the water-soluble fraction, produces a pronounced anestrous effect in mice. Our observations that neither the thyroid nor the pituitary exhibit histological changes following protracted *Lithospermum* feeding, do not necessarily militate against the suggestion by Cranston that the effect may be directly on the gonadotrophic elements of the anterior pituitary. It is certainly possible that an inhibitory effect may occur which is not of sufficient magnitude to elicit observable morphological changes. The fact that mice after 8 months of *Lithospermum*-induced diestrus immediately reestablish normal cyclicity when returned to normal diet, suggests that no permanent changes have occurred in any of the tissues involved. That the effect of *Lithospermum* results from a direct action

on the ovaries has been doubted by Cranston on the grounds that estrone injection immediately reestablishes cyclicity in *Lithospermum*-fed animals. It is possible that the *Lithospermum* effect is a threshold one of just sufficient magnitude to disrupt cyclicity, but insufficient to produce irreversible changes in the tissues involved in estrous cycles. Perhaps the use of a more potent concentrate of the active material would clarify this matter. There is the possibility, too, that the factor interacts with circulating gonadotrophins, although we have neither positive nor negative evidence of such action.

Summary. (1) The anti-estrous factor of *Lithospermum* appears to reside in the neutral water fraction. (2) The anti-estrous factor is most abundant in the flowers and seeds of the dried plant, and almost wholly absent from the stems. (3) Interference with estrus is stopped almost immediately, regardless of the length of treatment, when *Lithospermum* is withdrawn and animals returned to normal diet. (4) The C_3H strain is more sensitive to *Lithospermum* than is the Rockland Swiss strain. (5) The action of *Lithospermum* is unlike that of thiouracil. (6) The factor induces no observable changes in the anterior pituitary, thyroid, suprarenals, or pancreas; some atresia is observed in the ovaries, and some atrophy of the uteri.

16323

In vivo Observations on the Distensibility of the Femoral Venous System.*

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The marked engorgement of a vein when its central end is occluded tends to create the impression that veins are highly distensible structures. This illusion is probably created by the filling of partially collapsed veins[1] rather than by a true distension of the venous walls, since studies of segments of large veins[2,3] have revealed a relative lack of distensibility over the physiological range of venous pressures. The question arises as to whether observations on the large veins suited to direct distensibility measurements are necessarily typical of the venous system as a whole. Hochrein and Singer, for example, found the femoral vein considerably more distensible than the inferior vena cava.[2] Should the smaller veins and venules be highly distensible, alterations in venous pressure would be of considerable significance in altering the partition of the circulating blood volume between the venous system and other channels of the circulatory system.

Clark[4] attempted an estimate of the distensibility of the entire venous system of the forearm *in vivo* by employing a plethysmographic method. Her results were difficult to interpret, however, because of the inability to differentiate changes in venous volume from concomitant changes in tissue fluid volume. A more direct approach to this problem in the experimental animal is suggested by the simple observation that if the venous outflow from an organ is watched at the time the outflow pressure is suddenly lowered, the fall in pressure will be observed to produce a transient gush of excess blood. This excess blood obviously represents a volume passively flushed out of the system by

* Supported by a grant from the Life Insurance Research Fund.

1 Ryder, H. W., Molle, W. E., and Ferris, E. B., Jr., *J. Clin. Invest.*, 1944, **23**, 333; Duomarco, J., Rimini, R., and Predari, F. N., *Rev. Argentina Cardiol.*, 1946, **12**, 333.

2 Hochrein, M., and Singer, B., *Arch. Exp. Path. Pharmacol.*, 1927, **125**, 301.

3 Green, H. D., *Medical Physics*, Otto Glasser, Editor, Year Book Publishers, Chicago, 1944, 213.

4 Clark, J. H., *Am. J. Physiol.*, 1933, **105**, 418.

virtue of the reduction in the intravascular distending pressure. In a rigid system such a gush would be absent; in a highly distensible system it should represent a considerable volume. Although it is difficult to delimit the precise anatomical locus from which this excess blood arises, it must be predominantly from the venous system since a pressure change at the point of venous outflow from an organ could have very little effect on the pressures on the arterial side of the circuit. The present study represents an attempt to quantitate this phenomenon in the venous bed of the hind leg of the dog.

Methods. This analysis demanded an accurate method for measuring venous outflow. The simplest procedure is to open the appropriate vein and collect the outflow in a graduated cylinder for an accurately measured time interval. To be adaptable to the present study this method had to be made continuous so that the successive changes in rate of outflow produced by a change in the outflow pressure could be determined. This may be accomplished by determining the increase in volume in the collecting receiver gravimetrically. In preference to the rather cumbersome and frequently inaccurate mechanical devices that have been used for this purpose, we have developed a "strain gauge flowmeter" to obtain a continuous record of the increase in weight of the collecting vessel. This affords a small compact unit which may be placed directly under the receiving vessel and connected to the recording system by light flexible wires of any desired length. The resulting record is a quantitatively accurate graph of the cumulative venous outflow; any excess or deficit in this outflow produced by a change in outflow pressure may be measured directly on the original record.

We employ a gauge manufactured by the Statham Laboratories, Los Angeles, California. This is a self-contained Wheatstone bridge which is accurately balanced to null current when there is no stress applied to the pin actuating the sensitive resistance elements of the bridge. Although designed primarily for the measurement of minute displacements, these gauges may be used for the direct measurement of stresses in certain ranges. A gauge with a full range of eight ounces is well suited to measuring blood volumes of the order of 100 cc. The output of the gauge is sufficient to directly actuate optically recording galvanometers having periods somewhat less than 0.1 second.

In our application of this device, the outflow receiver (a beaker of 100 cc to 250 cc capacity) is placed on a small aluminum platform 8 x 10 cm. This platform is suspended on 3 points, 2 of which are steel pins pivoting on tapered bearings, and the third is the actuating pin of the strain gauge. The actual displacement of the platform over the full range of the gauge is so slight that frictional resistance in the suspension is of little consequence. To correct for variations in the mechanical advantage of the suspension due to alterations in the precise position of the beaker on the platform, we followed the practice of calibrating each record. This was accomplished by withdrawing an accurately measured volume (usually 20 cc) after each flow measurement and recording the resultant galvanometer deflection before the receiver had been moved from its recording position. Tests of the flow meter revealed almost complete linearity between galvanometer deflection and the volume (weight) in the collecting vessel, the deviation from linearity being 2.5% for the maximum range of the apparatus. By calibrating in the mid-range of each determination, this was reduced to an error of about $\pm 1\%$ and was disregarded.

The dogs were anesthetized with Na barbital (300 mg/kg) and heparinized. In preparing the hind leg, collateral circulation was excluded by severing all connections as high on the hip as possible except for the femoral artery, the femoral vein, the sciatic nerve, and the femur. The sciatic nerve was left intact and carefully protected with saline-soaked cotton to preserve some degree of vasomotor control. The femur was left intact to permit immobilization of the preparation without danger of kinking the blood vessels; collateral flow through the femur itself is negligible.[5] A cannula inserted dis-

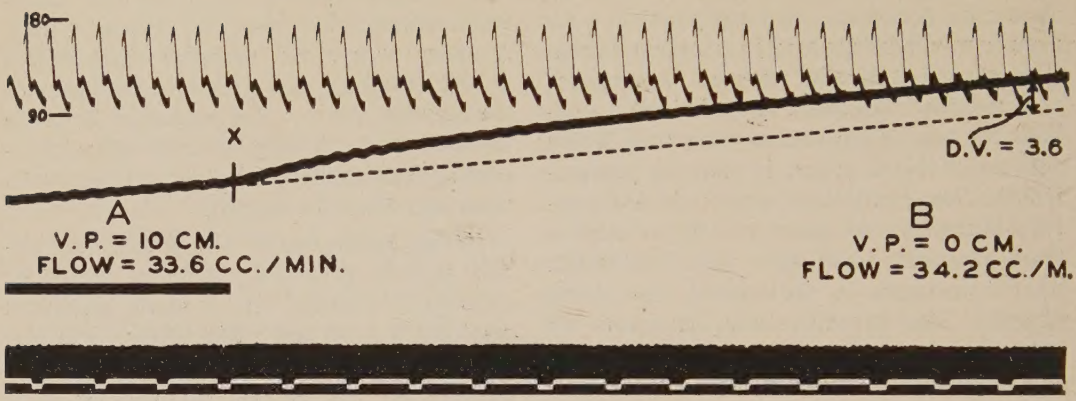

FIG. 1.

Record illustrating method of calculating distensibility volume (D.V.) from flow-meter tracing. At point "X" the venous outflow pressure was lowered from 10 cm to 0 cm. Time signal below indicates 1-second intervals.

tally into the femoral vein was connected by rubber tubing to an outflow orifice. A shunt circuit was provided to permit normal venous drainage of the leg into an external jugular vein between flow determinations as well as to permit return of the blood collected in the flow meter after each observation. The outflow orifice was a short glass tube mounted on a rigid vertical rod to which was attached a centimeter scale. The vertical rod was well lubricated and provided with suitable stops so that the outflow tube could be rapidly shifted from one hydrostatic pressure level to another with a minimum of mechanical jarring. To allow freedom of movement of this outflow tube, the connections with the vein represented a total length of 50 cm. The significant resistance to blood flow which might be introduced by connections of this length was avoided by making them of as large a bore as practical, the rubber tubing having a bore of 6 mm, and cannulae and connecting links having a bore of 4 mm. The outflow pressure as determined by the hydrostatic level of the outflow orifice was read directly in centimeters of blood, zero pressure reference being the approximate level of the vena cava.

The standard recording practice was to allow the flow to equilibrate at the initial outflow pressure and then record the rate of flow for a period of 10 seconds. The outflow pressure was then quickly shifted to the second level while the recording continued and in general a period of about 20 seconds allowed for equilibration of the flow at this new level. To maintain arterial pressure constant, a compensating reservoir was connected with a carotid artery and elevated to a hydrostatic level of 110 mm Hg. Most dogs of reasonable size would fill the dead space of the reservoir with about 200 cc of blood in equilibrating at this pressure, so that adequate blood was available to compensate for that collected in the flow meter and which was temporarily lost from the circulation. As a check on the arterial pressure perfusing the preparation, optical tracings of the pressure recorded from the opposite femoral artery were superimposed on the flow record.

— *Experimental Results.* The nature of the experimental recordings is illustrated in Fig. 1. The moment of pressure lowering (X) is followed by a transient steep rise which shortly equilibrates at a rate of flow (B) slightly in excess of the initial flow (A). In measuring the records, the assumption was made that at the moment the outflow pressure was lowered the arterial inflow changed to a value equal to the venous flow achieved after final equilibration at B. This assumption is not strictly correct because there will be a

[5] Green, H. D., Lewis, R. N., Nickerson, N. D., and Heller, A. L., *Am. J. Physiol.*, 1944, **141**, 518.

slight lag before pressure-flow relationships equilibrate at the new outflow-pressure level. Analysis of the outflow records with the thought of deriving a correction factor for this error, however, revealed that with changes in outflow pressure of only 10 cm H_2O the error was too small to warrant such an arbitrary correction. The gush of excess blood accompanying the sudden lowering of outflow pressure or what may be termed the "distensibility volume" (DV) may then be measured directly on the graph. For illustrative purposes this measurement is constructed graphically in Fig. 1; in actual practice the records were measured in reference to the time signal marks to correct for any variations in speed of the recording paper.

It is obvious that a comparable measurement may be obtained by the inverse procedure of suddenly elevating the outflow pressure, in which case there would be a momentary deficit in the outflow representing the volume of blood retained by the distension of the system at the higher pressure. Alternate determinations by the two procedures have yielded values in close quantitative agreement. We have selected the first method for routine observations merely because of the subjective satisfaction of seeing the distensibility volume as a positive increment.

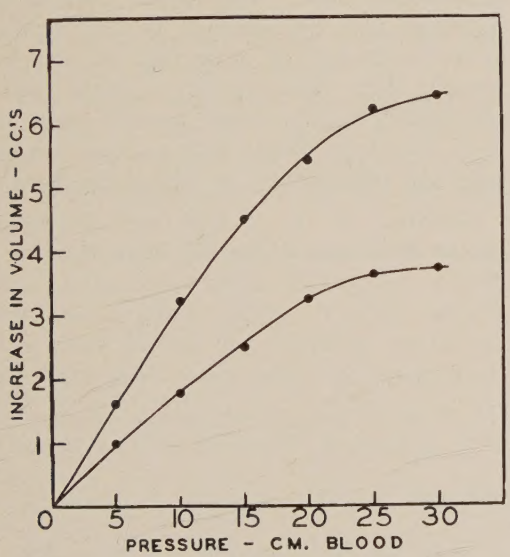

FIG. 2.

Plot of the data obtained from 2 of the dogs in the series showing the increase in volume of the venous bed of the hind leg produced by increases in the outflow pressure in the femoral vein.

In this fashion the distensibility volumes for 10 cm pressure changes in overlapping intervals for the range of 0 to 30 cm of blood have been determined. Simple addition of these individual values permitted plotting the data in the conventional volume-pressure manner. Two representative curves are shown in Fig. 2. It should be especially noted that the volumes given are those *in excess* of the volume in the system at zero pressure. It is unfortunate that this initial volume cannot be assessed by the present method.

Discussion. As an alternative to distensibility measurements on isolated segments of veins, the present method has a major advantage and an important limitation. Its advantage resides in the fact that it reveals the over-all effect of a change in the central venous pressure on the volume of blood pooled in the peripheral venous channels of the organ under study, and thus the values may be transferred directly to the interpretation of such changes in the circulatory dynamics of the intact animal. In contrast to direct observations on isolated veins, however, these data are of meager assistance in describing the precise physical properties of the system. This latter problem involves far more than the failure to define the exact anatomical limits of the distensible system under study. The pressure change produced at the central end of the femoral vein will not be paralleled by an equal pressure change throughout the femoral venous bed any more than it will produce an equal change in the femoral arterial pressure. On the contrary, it will represent an alteration in the normal venous pressure gradient, the change in this gradient being maximal at the outflow cannula and decreasing progressively as one traces the venous circuit back to the capillaries. It seems highly doubtful that a relatively small change in central venous pressure would significantly alter the pressure in the arterial portion of the capillaries if the arterial pressure head remains constant, a

relationship which justifies our use of the present method to study venous distensibility. However it also leaves in doubt a knowledge of the precise pressure changes produced in those segments of the venous bed which are yielding the distensibility volume when the central venous pressure is lowered. In addition it must be recognized that measurements of venous distensibility *in situ* are not determined solely by the properties of the vein walls, but may be influenced to some extent by the tension in extra-vascular supporting structures.[1]

The results obtained as illustrated in Fig. 2 reveal a volume-pressure relationship that exhibits the same trend as has been found typical of other blood vessels. The relationship is roughly linear over the pressure range of 0 to 20 cm of blood, the range which may be regarded as the range of normal physiological variation. Above this pressure the system appears to be approaching its elastic limit as the curves show a significant flattening in the 20 to 30 cm range. The actual magnitude of the distensibility volumes, moreover, is in accord with inferences derived from measurements on large veins which indicate a relatively low distensibility for the venous system. For a pressure change of 0 to 20 cm we observed an average distensibility volume of only 4.7 cc for the entire venous system in the hind leg of the dog as compared with an actual blood flow that averaged 31.3 cc/min. at an outflow pressure of 0 cm. As central venous pressure rises above this normal range the rigidity of the veins becomes even greater. This indicates that the venous system of the hind leg serves fairly efficiently as a network of pipes returning the blood from the periphery to the heart.

A complete evaluation of these distensibility characteristics would require information as to the total venous volume of the hind leg. We are not satisfied, however, that valid estimates of the average venous volume of the hind legs of mongrel dogs can be made with information or methods that are at present available. It should also be emphasized that the distensibility measurements reported here deal strictly with passive changes in venous volume without any consideration of changes in the volume of the venous bed which might be produced by venomotor action. There is ample evidence that active changes in the vein walls can occur,[6] but there is no conclusive evidence to demonstrate their significance *in vivo*.

Summary. The gush of excess blood that appears in the venous outflow from an organ when the outflow pressure is suddenly lowered has been selected as an index of the distensibility of the venous system of that organ. With the aid of a new type of flow meter to quantitate venous outflow, this phenomenon has been studied in innervated hind legs of anesthetized dogs. The results indicate a relatively low distensibility in the venous system of the hind leg of the dog; the volume-pressure data demonstrating a fairly linear relationship in the venous pressure range of 0 to 20 cm of blood, with a trend toward even less distensibility at higher pressures.

[6] Franklin, K. J., *A Monograph on Veins*, Charles C. Thomas, Springfield, Ill., 1937.

16324

Absence of Prolongation of Pseudopregnancy by Induction of Deciduomata in the Mouse.*

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The similarity of the deciduomal reaction to the maternal placenta has prompted several investigations of the possibility that phenomena associated with pregnancy might be found in animals bearing deciduomata. Hammond[1] observed that the mammary development of the pseudopregnant rabbit was not enhanced by the presence of the reaction. Similar results were obtained by Nelson[2] who found also that surgical removal of the deciduomata did not initiate lactation. More recently, however, Ershoff and Deuel[3] have reported that although the presence of deciduomata did not effect the mammary development of the pseudopregnant rat, their presence was associated with a marked delay in the recurrence of estrous vaginal smear. In the normal untreated rat the duration of pseudopregnancy averaged 13.7 days; if deciduomata were induced by traumatizing the uterus on the fourth day, estrus did not recur before the twenty-second day.

The present experiment was performed to determine whether the induction of deciduomata in the mouse would prolong the duration of pseudopregnancy as has been reported in the rat.

Experimental. Twenty young adult female mice of the Swiss albino strain were mated with vasectomized males. Vaginal smears were made on the day following copulation and were taken daily until the appearance of a completely cornified smear indicated the recurrence of estrus. A control group of 10 mice received no further treatment. In the remaining 10 animals deciduomata were induced by placing longitudinal sutures in the lumina of each uterine horn on the third day after mating. At the end of the experiment these animals were autopsied and the uteri examined grossly to ascertain the presence of deciduomal responses.

In the untreated pseudopregnant mice estrus recurred 11.3 ± 0.68 days after mating; in the group of animals in which deciduomata had been induced, estrus recurred 10.7 ± 0.46 days after mating.

Discussion. The present results show that the presence of deciduomata has no significant effect on the duration of pseudopregnancy in the mouse. The marked prolongation of pseudopregnancy in the rat under similar circumstances[3] indicates a decided species difference. The nature of the physiological processes involved is not clear, since in most respects the two species exhibit very similar reproductive phenomena. A clue may be provided, however, in the report of Atkinson[4] that in the mouse deciduomata induced on the third day of pseudopregnancy persist intact only until the seventh day and then rapidly degenerate. In the rat, on the other hand, Ershoff and Deuel[3] report that the deciduomata persist intact until the fourteenth day. Since it has been shown that the persistence of the deciduomal reaction is dependent on the presence of a high level of progesterone,[4] we may assume that the prolongation of pseudopregnancy in the rat is the result of prolonged luteal activity. The mechanism by which this is effected in the

* Aided by a grant, administered by Dr. Philip E. Smith, from the Rockefeller Foundation, New York.

1 Hammond, J., *Proc. Royal Soc. B,* 1917, **89**, 534.

2 Nelson, W. O., *Anat. Rec.,* 1932, **54** (suppl.), 50.

3 Ershoff, B. H., and Deuel, H. J., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 167.

4 Atkinson, W. B., *Anat. Rec.,* 1944, **88**, 271.

rat but not in the mouse must remain unexplained at the present time.

Summary. Unlike the rat, the induction of deciduomata does not prolong pseudopregnancy in the mouse. The mechanism responsible for this marked species difference in response to apparently identical physiological conditions is obscure.

16325

Circulating Antibodies in Vitamin Deficiency States: II. Thiamin and Biotin Deficiencies.

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Introduction. In the first paper of this series,[1] we presented data concerning the effects of pyridoxin, pantothenic acid, and riboflavin deficiencies upon antibody production by the rat in response to human red blood cells as antigenic stimulus. The present paper deals with the effects of thiamin and biotin deficiencies upon antibody production under similar experimental conditions.

Experimental. Male weanling albino rats of the Sprague-Dawley strain were distributed as indicated in Table I. The animals were housed individually in wide-mesh, screen-bottom cages and weighed weekly. Both the thiamin-deficient rats and their controls received a basal diet with the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,[2] 4.00; cod liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; *i*-inositol, 0.03; and 2-methyl-1, 4-naphthoquinone, 0.001. For the biotin-deficient rats and their controls, this diet was modified by replacing 60% of the casein with dried egg white. All rats received additional vitamins in the form of a daily pill. Each of the pills given to the 2 control groups supplied the following vitamins: thiamin, 40γ; riboflavin, 60γ; calcium pantothenate, 200γ; pyridoxin, 50γ; biotin, 4γ; folic acid, 1γ; nicotinic acid, 100γ; and *p*-aminobenzoic acid, 1mg. For the thiamin- and biotin-deficient groups, the appropriate vitamin was omitted from the pill. The basal diets were fed *ad libitum* to the 2 deficient groups, while the daily food intake of each rat in the control groups was restricted to that consumed during the previous day by its paired member in the corresponding deficiency group.

In Series I, immunization of the thiamin-deficient rats and their controls was begun after 3 weeks on experiment. At this time, the animals were progressively losing weight. Three of the controls and 2 of the deficient rats died during the immunization period. Because of this high mortality, this experiment was repeated exactly in Series II and immunization was instituted after 2 weeks. Although a similar weight loss occurred, no mortality was noted. The biotin-deficient rats and their controls of Series I were immunized after 6 weeks on experiment. At this time, the deficient animals had plateaued in weight and exhibited the typical symptoms of biotin deficiency, *i.e.*, alopecia, dermatitis, blepharitis, and cheilosis.

A 10% suspension of washed, Group O, Rh positive human erythrocytes in normal saline was given intraperitoneally as antigen. An initial dosage of 0.5 ml of the red cell suspension was followed by 2 one cc injections. Inoculations were made on alternate days. Five days after the final injection the

[1] Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 137.

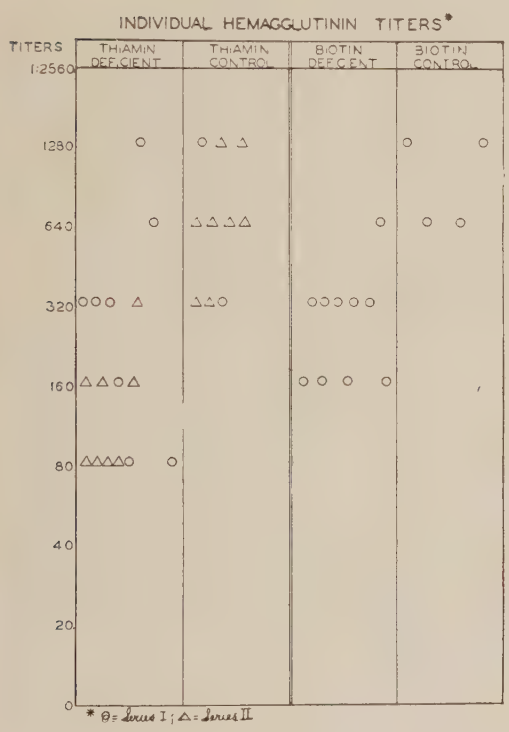
[2] Jones, J. H., and Foster, C. J., *J. Nutrition*, 1942, **24**, 245.

TABLE I.
Summary of Growth and Food Consumption Records.

Series	Group	No. of rats	Body weight*		Daily food consumption*
			Initial	Final†	
I	Thiamin-deficient	8	42	41	2.9
	Thiamin control	2	41	55	2.9
	Biotin-deficient	10	43	176	9.6
	Biotin control	4	44	218	9.6
II	Thiamin-deficient	8	61	64	4.4
	Thiamin control	8	61	70	4.4

* Group avg in g.
† At the time of bleeding.

TABLE II.



rats were bled and the serums tested for agglutinin titer as previously described.[1] Serums of rats from our stock colony possessed no agglutinins for human Group O, Rh positive red blood cells.

Results. The individual hemagglutinin titers are recorded in Table II. It is evident that the content of circulating antibodies in the thiamin and biotin deficient rats was less than that of the control rats. It is of interest that the decreases noted in the present work are not as marked as those previously observed, in pyridoxin and pantothenic acid deficiencies.[1] It would seem, therefore, that biotin and thiamin are not as critical as pyridoxin and pantothenic acid for optimal antibody response.

Stoerk, Eisen and John[3] and Ruchman[4] failed to observe any influence of a thiamin deficiency upon antibody response in the rat. A direct comparison of our results with those of these workers is difficult because of the variance in experimental procedures.

Summary. (1) Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in thiamin- and biotin-deficient rats. (2) A moderate impairment of antibody response was observed in both deficiencies.

[3] Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

[4] Ruchman, I., *J. Immunol.*, 1946, **53**, 51.

16326

Wound Healing in Rats with Biotin, Pyridoxin, or Riboflavin Deficiencies.

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Retardation of wound healing due to deficiency of vitamin C has long been recognized and the cytologic alterations in wounds consequent to such deficiency have been described. Hunt[1] demonstrated tinctorially that the primary defect of wounds in scorbutic guinea pigs is the immaturity of the intercellular matrix produced by fibroblasts. The role which vitamin deficiencies other than that of vitamin C may play in wound healing has not been adequately studied.

It is the purpose of the present communication to record qualitative and quantitative differences in the healing of 240 wounds in 60 young rats of uniform strain, evenly distributed to include normal controls and those with deficiency of either riboflavin, pyridoxin, or biotin.

Methods: Care of Animals. Weanling, male, albino rats from the Sprague-Dawley strain were divided into 4 groups of 15 each. One group, serving as *controls,* was fed a basal diet of the following composition: sucrose 76, Labco casein ("vitamin-free") 18; salts[2] 4, and corn oil 2. Each rat received the following vitamins daily in a supplement dish: thiamin HCL 30γ, pyridoxin HCl 30γ, riboflavin 30γ, nicotinic acid 250γ, calcium pantothenate 100γ, choline chloride 10 mg, and *i*-inositol 3 mg. The *riboflavin-* and *pyridoxin-deficient groups* received the same basal diet and supplements as the controls, except for the omission of riboflavin and pyridoxin, respectively. For the *biotin-deficient* group the basal diet was modified by substituting 56% of the casein by powdered egg white. The vitamin supplements were identical with those of the control group.

The animals were housed in individual cages with wide-mesh screen bottoms and the basal diets were fed *ad libitum.* Each rat was given 2 drops of Abbott's haliver oil (plain) containing 1.3 mg of added d.l*a*-tocopherol acetate, weekly, by mouth. The rats were weighed each week.

Production and Characterization of Wounds. After 5 weeks on the respective diets, standard wounds (Series I) were produced on the lateral aspects of each thigh in the following manner: By means of a special stamp a circle 13 mm in diameter was outlined upon the shaved skin with carbol-fuchsin. Under ether anesthesia, by carefully following the outline of this circle with an electric knife, a button of skin extending down to the loose fascia was removed. The intensity of the current was such that the cautery effect did not extend more than one mm beyond the line of excision. No attempt was made to establish aseptic conditions or to cover the wounds. The resulting ulcers, together with a rim of apparently normal skin, were excised under ether anesthesia 3, 6, 14, or 20 days later. The skin edges were approximated with cotton sutures after excision of the ulcers. The excised ulcers were fixed in formalin.

A second series of wounds was made in the same animals 3 weeks following the production of the first series. The procedure was identical with that of the first series, but the site of the wounds was now on both lateral sides of the chest. The lower border of the wounds was over the last rib. Three animals from each group were sacrificed at 3 and at 6 days, respectively. The animals were placed in formaldehyde solution, and the ulcers excised after fixation. Five of the remaining animals in each group were sacrificed at 14, and the other 4, at 20 days, and the ulcers similarly excised.

[1] Hunt, A. H., *Brit. J. Surg.*, 1941, **28**, 436.

[2] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

Paraffin sections were prepared from a block which was cut as nearly as possible through the center of the ulcer. These sections were stained with hematoxylin and eosin, a combination of the Van Gieson and the Weigert elastic tissue stains, and the Wilder reticulum stain.

The healing process in each section was studied with regard to the following characteristics:

1. Demarcation and separation of devitalized tissue.
2. The epithelialization of the ulcer.
3. The relative amount of collagen in the scar tissue, as indicated by pink fibers in the Van Gieson, and non-argyrophilic fibers in the reticulum stain.
4. The relative amount of precollagen in the scar tissue, as indicated by yellow fibers in the Van Gieson, and argyrophilic fibers in the reticulum stain.
5. The density of the scar tissue.
6. The vascularity of the scar tissue.
7. The cellular infiltration of the scar tissue.

Results. Observations on wounds of Series I and II are combined since significant differences between the two series were not observed.

General Effects of Dietary Deficiencies. After 5 weeks on the respective diets (the time at which the first wounds were inflicted) the average weights were as follows: biotin-deficient group, 127 g; pyridoxin-deficient group, 82 g; riboflavin-deficient group, 59 g; and control group, 176 g. The average weights of the vitamin-deficient groups remained fairly constant thereafter, whereas that of the control group continued to increase throughout the experiment. Besides the retardation in growth, the animals receiving vitamin-deficient diets developed the symptoms characteristic of the respective deficiencies. These symptoms were more severe during the healing of the second wounds than they were while the first wounds were healing.

Gross Observations of Wounds. At no time was there gross evidence of infection. All wounds remained dry and clean. The ulcers of the control and biotin-deficient groups appeared to heal equally rapidly, while the healing of the ulcers of the pyridoxin- and riboflavin-deficient groups was strikingly delayed. There was also a considerable difference in the thickness of the skin of the rats, detectable by palpation. The skin of the control and biotin-deficient groups was moderately thick, in contrast to the thin skin of the other two groups (confirmed by microscopic measurement).

Toward the end of the first week following the production of the wounds, the ulcers of the control and biotin-deficient groups were definitely smaller than those of the other two groups. Since microscopic section at this time showed very little epithelial or connective tissue growth, the difference in the size of the ulcers could be explained only on the basis of a difference in the contraction of the skin about the ulcers.

The ulcers of the control and biotin-deficient groups were healed at 14 days and those of the pyridoxin- and riboflavin-deficient groups, at 20 days.

Microscopic Observations of Wounds (see Fig. 1). An early reaction in the wounds is a narrow zone of leukocytic infiltration which forms at the junction of devitalized tissue (from the cautery effect of the cutting current) with viable tissue. This leukocytic zone of demarcation extends from the epidermis to the base of the ulcer. Epithelium regenerating from the surface and from sweat glands and hair-follicles adjacent to the leukocytic zone grows immediately subjacent to the latter. Later a cleft develops in the leukocytic zone which marks the beginning separation of this devitalized tissue. Because of the similarity of this process to the sequestration of necrotic bone, it will hereafter be referred to as sequestration. The separating, necrotic, burned tissue becomes an integral component of the eschar, together with dried serum, condensed fibrin, blood, and cellular exudate.

Biotin-deficient group. During the first 2 weeks there is some retardation in collagen production and in the density of the granulation tissue, while the amount of precollagen

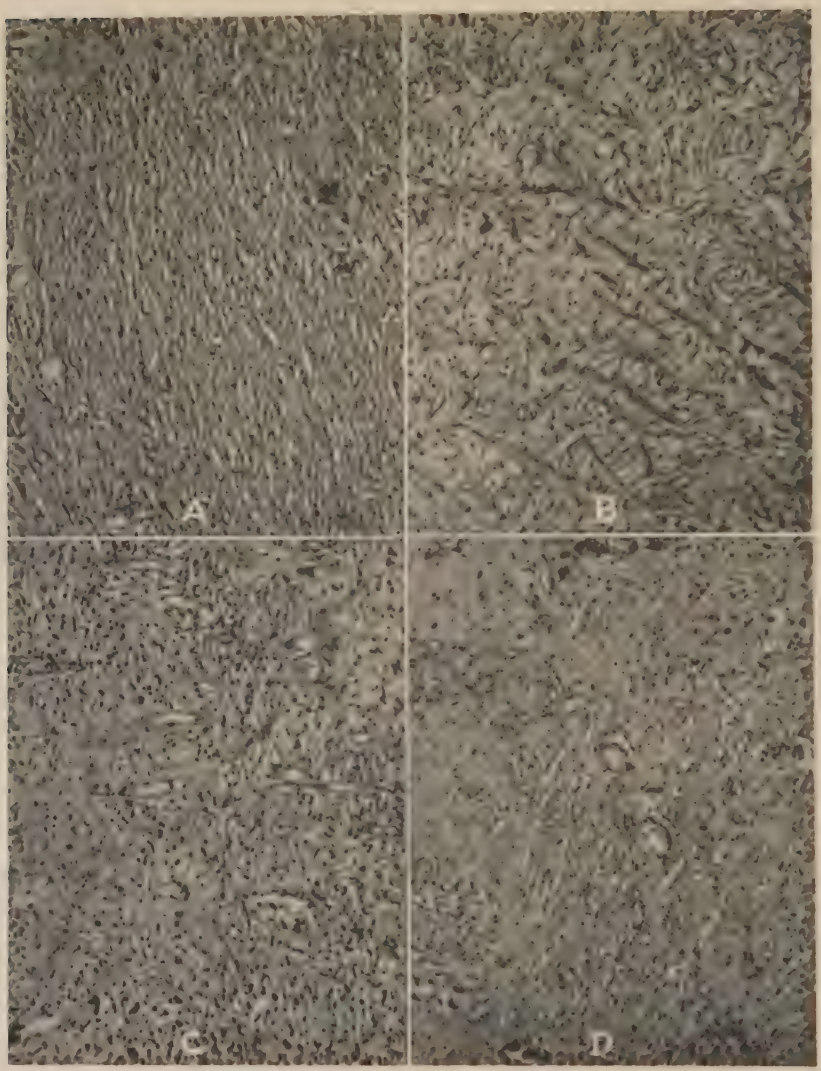

FIG. 1.
Photomicrographs of representative areas of 14-day-old wounds stained with a combination of the Van Gieson and the Weigert elastic tissue stains, × 130. A is from a control rat; B, from a riboflavin-deficient rat; C, from a pyridoxin deficient rat; and D, from a biotin-deficient rat.

and the vascularity are greater than is seen in the normal animal. At 20 days the scar is identical with that of the control.

Pyridoxin-deficient group. Initially there is retardation of leukocytic reaction and in the development of granulation tissue. Epithelialization is delayed throughout. Although terminally these ulcers are epithelialized as well as the control ulcers, the scars contain less collagen and a considerable amount of precollagen. The latter is absent at this time in the control and biotin-deficient groups. The scar tissue is less dense, more vascular, and more cellular than that of the control group. During the course of healing there is retardation of four to seven days in the evolution of the lesion, as compared with that of the control group.

Riboflavin-deficient group. There is delay not only in the evolution of the granulation tissue, but also in the epithelialization of the ulcer. Granulation tissue is tardy in appear-

ance and terminally it contains a decreased amount of collagen with considerable admixture of precollagen. The scar is less dense than that of the control group, but more dense than that of the pyridoxin-deficient group. The vascularity and cellularity are increased.

Discussion. Disturbance in the production of collagen is generally considered characteristic of scurvy, but is also known to occur in hypoproteinemia. The results obtained in the present experiments indicate that a similar disturbance is associated with deficiency of biotin, pyridoxin, or riboflavin in rats. However, the retardation of collagen production in these vitamin-deficiencies is less pronounced and less persistent than in scurvy.

The pyridoxin- and riboflavin-deficient groups exhibit, in addition, delayed contraction of the wound as observed grossly, and tardy exudation; the latter resulting in delayed sequestration and production of granulation tissue. The residual increased vascularity and correlative decreased density of the scars of the pyridoxin- and riboflavin-deficient groups are further indications of retardation in wound healing which are logically associated with the terminally decreased collagen and increased precollagen.

The wound of biotin-deficient animals exhibits a somewhat diminished density and decreased collagen content of the scar. There is no retardation of exudation or of granulation tissue production. Terminally the scar is indistinguishable from that of a normal animal.

The role which inanition plays in the retardation of wound healing is difficult to assess. It may or may not be coincidence that the greatest retardation in wound healing occurs in animals where inanition is also maximal—in the riboflavin- and pyridoxin-deficient groups. However, while the retardation in wound healing is greater in pyridoxin-deficient rats than in the riboflavin-deficient animals, inanition is greater in the latter than in the former.

Summary. The process of wound healing has been observed in rats exhibiting severe deficiency of biotin, pyridoxin, or riboflavin. Marked impairment of rate and quality of healing was noted in the pyridoxin- and riboflavin-deficient groups. The biotin-deficient group showed only mild delay in healing.

We are indebted to Merck and Company, Inc., for the B vitamins used in these studies.

16327

Peptidases in Human Serum.*

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(Introduced by J. S. L. Browne.)

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Although the existence of proteolytic enzymes in blood serum and leucocytes has long been recognized, no definitive work has been done in this field until recent years (cf. review of the early literature, Opie[1]).

With the development of the use of synthetic di- and tri-peptides as substrates, a quantitative microtitration technique was devised for the determination of peptidase hydrolysis (Grassmann and Heyde,[2] Abderhalden and Hanson,[3] Maschmann,[4] Fruton,[5]

* This study was aided by a grant from the John and Mary Markle Foundation.

1 Opie, E. L., *Physiol. Rev.*, 1922, **2**, 552.

2 Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1929, **183**, 32.

3 Abderhalden, E., and Hanson, H., *Fermentforsch.*, 1937, **15**, 382.

4 Maschmann, E., *Biochem. Z.*, 1941, **308**, 359.

5 Fruton, J. S., *J. Biol. Chem.*, 1946, **166**, 721.

TABLE I.
Hydrolysis of LGG, GL, and GG by Normal Human Serum.
(The hydrolysis of LGG is most rapid and considerably activated by cobalt ions.)

Substrate .05 M	cc human serum per cc test sol.	Metal ion	Hydrolysis *% per hour
LGG	.4	——(avg of 5)	7.3
	.4	Mg (.01 M)	5.0
	.4	Mn (.001)	5.5
	.4	Co (.001)	9.6
GL	.4	——(avg of 4)	2.3
	.4	Mg (.01 M)	1.2
	.4	Mn (.001)	3.4
	.4	Co (.001)	2.5
GG	.4	——	0.0
	.4	Co (.001 M)	3.0

* % per hour of the hydrolysis expected on the complete splitting of one peptide linkage.

and Holman *et al.*[6]). Except for some preliminary observations on pathological human serum with l-leucylglycylglycine as substrate (Grassmann and Heyde[7]) the method has found no clinical application thus far.

A study was undertaken to investigate proteolytic activity of the human serum in clinical conditions associated with ageing. As a preliminary step, however, it was necessary to study this function in normal control subjects and under various conditions involving leucocytosis and tissue proliferation.

Method. Blood was obtained from nonfasting subjects by venous puncture. Serum was prepared by centrifugation.

The tri-peptide l-leucylglycylglycine (LGG) and the di-peptides glycyl-l-leucine (GL) and glycylglycine (GG) were used.† In the activation studies the method described by Maschmann[4] was followed, in which 0.01 M cobalt, 0.01 M manganese or 0.1 M magnesium were added as sulphates. The reaction was carried out in a water bath at 39°C in 2.0 ml volumetric flasks containing 0.05 mM/cc of the substrate. The pH was maintained near pH 7.8 with 0.01 M phosphate or veronal buffer, both of which were found to be equally effective. 0.01 ml of toluene was added to each tube as preservative. In the preliminary experiments on LGG, GG and GL, 0.4 cc of serum per ml of reaction mixture were used. With cobalt as activator, the concentration of serum was reduced to 0.2 cc per ml of test solution in the studies on LGG. The experiments on the 11 normal control subjects and the pathological cases were carried out on LGG with this serum concentration. The rate of proteolytic hydrolysis was followed in triplicate according to the titration method of Grassmann and Heyde.[2] Four readings were taken over a period of 7 hours, following a zero time determination. The results were corrected for serum and substrate controls which were run simultaneously. The rate of activity per hour was calculated from the slope of the zero order plot of the per cent hydrolysis versus time (per cent hydrolysis equals per cent of the hydrolysis expected on the complete splitting of one peptide linkage).

Subjects. Eleven members of the staff (5 males and 6 females) ranging in age from 24 to 47 years were studied as normal controls. Investigations were carried out on 10 subjects suffering from various pathological conditions. These included dental extraction, postpartum infections, fractures, aleukemic leukemia, cancer and non-malignant tumor.

Results. The results are presented in Tables I and II. In the serum of 11 normal control subjects the rate of hydrolysis ranged from 6.1 to 7.8%/hour with an average of $7.2 \pm 0.78\%$.

[6] Holman, H. R., White, A., and Fruton, J. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 196.

[7] Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1930, **188**, 69.

† These peptides were obtained through the courtesy of Dr. M. S. Dunn at the University of California in Los Angeles.

TABLE II.
Action of Serum Peptidases in Various Abnormal Conditions.*

Diagnosis	Condition	Age	Sex	Hydrolysis % per hr
Vulvar carcinoma, epilepsy	Sed. rate 39	39	F	9.4
Cervical carcinoma	" " 30	47	F	5.2
" "	" " 52	52	F	10.9
Non-malignant tumor	" " 49	46	F	6.4
Aleukemic leukemia	WBC: 6,500/cu mm Lym.: 4,680/cu mm High sed. rate	47	M	7.4
Postpartum pneumonia	6th day postpartum	23	F	11.0
	10th " "			7.6
" infection	Penicillin treatment 10th day postpartum	29	F	9.6
Leg fracture	Fever 4th day after fracture	58	M	13.0
" "	Day of fracture	62	M	12.6
Lumbar and arm fracture	Day of fracture	35	F	14.6
	6th day			8.4
Dental extraction	1 day after	26	F	5.5

* The reaction mixture consisted of 0.05 M LGG, 0.001 M cobalt, 0.01 M veronal or phosphate buffer; 0.2 cc of serum were used per cc of reaction mixture.

The manner of compiling the data is illustrated in Fig. 1.

Discussion. The present investigation shows that there exists in human serum a cobalt-activatable LGG-splitting peptidase. The rate of hydrolysis is uniform in healthy adult subjects and there are considerable deviations from this normal range in various pathological conditions. The results obtained in this study (Table I) agree with the data of Grassmann and Heyde[7] on human serum. Fruton[5] calculated from this data a value of 0.035% hydrolysis per minute (K°LGG) for 0.1 ml of serum per ml of test solution. Since we found direct proportionality between serum concentration and rate of reaction,‡ our data yields a corresponding K°LGG of 0.030±0.01.

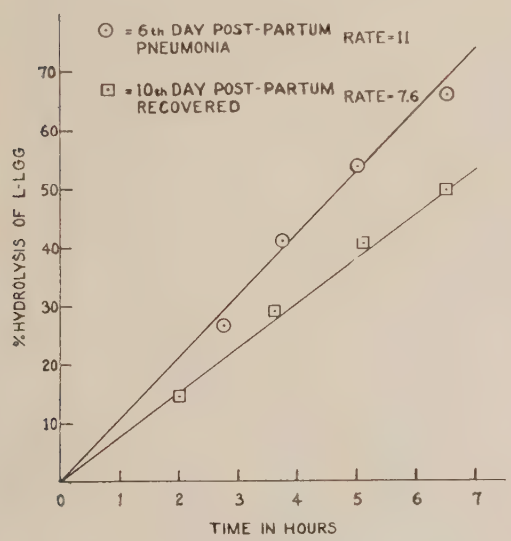

FIG. 1

Hydrolysis of LGG by serum from a case of postpartum pneumonia. It may be seen that the kinetics of the reaction are of a zero order.

From our limited number of cases more detailed conclusions can be drawn only with great reservation. The fact that the rate of hydrolysis was highest in cases of puerperal infection and fractures, and decreased with the progress of healing, suggests further studies along the lines of tissue reaction and repair. This is also indicated by the observations of Zamecnik *et al.*[8] who reported a rise of serum peptidases in animals following burns.

Summary. The rate of hydrolysis of l-leucylglycylglycine by the cobalt activatable peptidase in human serum shows little variation in normal adults, but is increased in certain pathological states, particularly in fractures and postpartum infections.

‡ Unpublished experiments.

8 Zamecnik, P. C., Stephenson, M. L., and Cope, O., *J. Biol. Chem.*, 1945, **158**, 139.

16328

A Photometric Adaptation of the Zinc Uranyl Acetate Method for Sodium.

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Several colorimetric methods suitable for use with biological material have been proposed for the determination of sodium.[1,2,3] The disadvantage of these in routine biochemical analysis varies from a lack of the necessary sensitivity[1,2,3] to interference from chromogenic materials usually encountered.[3]

The proposed method is a modification of that of Butler and Tuthill.[4] All reagents used are described in that method. The precautions and stoichiometric relations are carefully preserved while the tedious weighing and washing procedures have been eliminated. In addition, a greater sensitivity is obtained which makes possible the use of a smaller volume of sample and the analysis of fluids with very low sodium content.

The procedure will be described for urine. Serum and other fluids may be prepared for analysis according to Peters and Van Slyke[5] with appropriate dilution to bring the solutions into the range of the standard curve.

Method. Add to 5 ml of urine in a 50 ml volumetric flask, 5 ml of 20% trichloroacetic acid. Mix, and dilute with water to the mark. After again thoroughly mixing, filter out any precipitated protein, if present, using a dry Whatman No. 40 or 42 filter paper and discarding the first 5 ml of filtrate.

To 10 ml of uranyl zinc acetate reagent in a 15 ml graduated pyrex centrifuge tube, add dropwise 1 ml of the urine filtrate (or prepared sample of serum, etc.) with constant stirring. Mechanical stirring is desirable though not imperative. Allow the mixture to stand for 30 to 45 minutes with occasional stirring. Remove the stirring rod, washing down with 3 ml of reagent as it is being removed. Cap the tubes (IEC No. 580 rubber caps) and centrifuge at 2000 r.p.m. for 5 to 10 minutes. Aspirate off the supernatant fluid as completely as possible. Wash the precipitate with 95% ethyl alcohol saturated with sodium zinc uranyl acetate, cap and centrifuge as above. Aspirate the supernatant and repeat the washing and aspiration. After drying the tubes in an oven or water bath at 60° to 70°C for 5 to 10 minutes, add 8 ml of hot water (60°C) to dissolve the precipitate. Cool to room temperature, make up to the 10 ml mark with water and mix. Bring the temperature to 25°C in a water bath and read at 430 mμ in a spectrophotometer or with a suitable blue filter in any photoelectric colorimeter. The zero density setting is made using a reagent blank of 1 ml water treated and washed in the same manner as the sample. The concentration of the unknown is read from a standard curve prepared with sodium chloride solutions containing 0.002 to 0.05 meq sodium/ml.

Precautions. 1. Stirring of the solution should be continued for at least 1 minute after the precipitate appears. Incomplete stirring will give erroneously low results.

2. Aspiration of the supernatant fluid in the centrifuge tube is preferable to decantation because the granular quality of the precipitate is such that it could easily be lost through rough handling. It is obvious that as much as possible of the supernatant fluid should be removed with each aspiration. This is best done if a drawn out capillary tube is used as an aspirator.

3. In the washing procedure it is best to

* Aided in part, by a grant from the United States Public Health Service.

1 Salit, P. W., *J. Biol. Chem.*, 1932, **96**, 959.

2 Arnold, A. E., and Pray, A. R., *Ind. and Eng. Chem.* (Anal Ed.), 1943, **15**, 294.

3 Bradbury, J. T., *J. Lab. Clin. Med.*, 1946, **31**, 1257.

4 Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

5 Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Baltimore, Williams and Wilkins, 1932, p. 733.

stir up the precipitate by forcing the alcohol wash solution over it in a sharp stream. The last 1 to 2 ml of the wash solution should be used to wash down the sides of the centrifuge tube.

Discussion. It will be noted that in the preparation of the urine sample, as given above, a 1:10 dilution of urine is prepared and that the phosphate has not been removed. We have found only negligible differences in comparative values obtained whether or not phosphate is removed at this dilution. An advantage offered by the extreme sensitivity of this method is that samples of very low sodium content are immediately recognized at the first step in the precipitation; *i.e.*, a minute amount of precipitate forms. In such cases, it usually suffices to repeat the precipitation using the preparation as recommended by Butler and Tuthill in which the urine is not diluted during the removal of protein and phosphate.

In the determination of serum sodium, a 1 ml aliquot of the solution of ashed sample containing the equivalent of 0.1 ml of serum is used. Here, again, the great dilution eliminates the necessity for the removal of phosphate.

Recoveries of sodium added to urines of known value are shown in Table I. Comparison of results obtained with the proposed method and that of Butler and Tuthill are presented in Table II.

The color characteristics of the water solution of the sodium uranyl zinc acetate complex were determined using both a Beckman Model DU Spectrophotometer and a Coleman Model 11A Spectrophotometer. Absorption maxima were obtained at 430 mμ on both instruments. Plots of concentration *vs* density do not give a straight line at this wave length. However, the hyperbolic curve obtained is smooth and highly reproducible.

The yellow color of the dissolved complex is stable for at least 4 hours. The intensity varies with temperature, and unknown solutions must be read at the same temperature at which the standard curve was prepared.

TABLE I.
Recovery of Sodium Added to Urine Samples.

Meq. sodium added	Meq. sodium recovered	% recovered
.0118	.0113	95.8
.0118	.0116	98.3
.0059	.0055	93.3
.0059	.0057	96.6
.0020	.0020	100.0
.0020	.0021	105.0

TABLE II.
Comparison of Results with Colorimetric and Gravimetric[4] Procedures.

Gravimetric—Meq./liter	Colorimetric—Meq./liter
36.0	36.0
69.0	68.8
28.6	30.0
63.6	65.0
82.3	81.4
89.0	87.5

In explanation of the sensitivity obtained by this method, it may be suggested that the intrinsic errors in weighing and possible imperceptible small leakage through the pores of the sintered glass filter are additive factors in the gravimetric procedure, which are eliminated in the procedure described.

Summary. A photometric method is described based on the gravimetric procedure of Butler and Tuthill in which the precipitated sodium zinc uranyl acetate is dissolved in water, giving a yellow color which is read with a suitable filter against a standard curve at 430 mμ. Phosphates in the normal urine were found to exert negligible interference. Recoveries and comparison values show the method to be more rapid and at least of equivalent accuracy with the gravimetric procedure.

I wish to express my appreciation to Professor C. J. Farmer and Dr. Edith B. Farnsworth for their aid in carrying out this study, and to Miss Lora Belle Hughes who performed most of the technical work.

16329

Tuberculostatic Action of Chloromycetin *In vitro* and *In vivo*.*

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Ehrlich, Bartz, Smith, Joslyn and Burkholder[1] reported the isolation of a *Streptomyces sp.* which produced a new antibiotic which they named Chloromycetin. This antibiotic was highly bacteriostatic *in vitro* for a variety of bacteria, but only moderately bacteriostatic for the virulent H37Rv strain of *M. tuberculosis*. Chloromycetin showed considerable activity against *Rickettsia prowazeki* in tests using chick embryos. Smadel and Jackson[2] have also reported that chloromycetin was an effective chemotherapeutic agent experimentally against a variety of Rickettsiae. In a recent, more detailed report Smith, Joslyn, Gruhzit, McLean, Penner and Ehrlich[3] have further described the properties of chloromycetin, including data on toxicity and chemotherapeutic activity. The *in vitro* tests of the bacteriostatic activity of chloromycetin for *M. tuberculosis var. hominis* (H37Rv) reported in the above publications were done in this laboratory. This work has now been extended to include *in vitro* tests on other strains of virulent human-type tubercle bacilli and the effect of chloromycetin administered subcutaneously and orally on experimental murine tuberculosis.

Methods. The *in vitro* bacteriostatic tests with chloromycetin† were performed by the technic previously reported[4,5,6] and the results recorded in terms of the least amount of chloromycetin which would completely inhibit the subsurface growth of 0.01 mg of tubercle bacilli per ml of synthetic medium. Duplicate tests were conducted in the same medium to which had been added enough sterile beef serum to make a final concentration of 10.0%. Nineteen strains of virulent human-type tubercle bacilli were employed, including 7 which were resistant to more than 1000.0 μg of streptomycin per ml. One bovine strain was also used. Similar *in vitro* tests were conducted in which chloromycetin was employed in combination with streptomycin and para-aminosalicylic acid (PAS).

The chemotherapeutic action of chloromycetin *in vivo* was determined by using mice infected intravenously with 0.1 mg of the H37Rv strain of *M. tuberculosis*. The technic employed in these tests and the evaluation of the results of the tests were in every respect similar to those previously reported employing streptomycin and PAS,[6,7] with the exception that chloromycetin, when administered subcutaneously to mice, was dissolved in 20% propylene glycol water solution and the experiments were terminated at the end of 21 days instead of 28 days.

Results. The results of the *in vitro* bacteriostatic tests with and without serum using the 19 human type strains and the one bovine strain are shown in Table I. There was as much as an 8-fold variation in the sensitivity of these strains to chloromycetin when serum was omitted from the medium. Since twofold dilutions were used, the majority of the strains (12 out of 19) required

* Work aided by a research grant from Parke, Davis & Company, Detroit, Mich.

1 Ehrlich, John, Bartz, Quentin R., Smith, Robert M., Joslyn, Dwight A., and Burkholder, Paul R., *Science*, 1947, **106**, 417.

2 Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

3 Smith, Robert M., Joslyn, Dwight A., Gruhzit, Oswald M., McLean, Wm. I., Jr., Penner, Mildred A., and Ehrlich, John, *J. Bact.*, 1948, **55**, 425.

† Crystalline chloromycetin obtained from Parke, Davis Company, Detroit, Mich.

4 Youmans, Guy P., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 119.

5 Youmans, Guy P., and Doub, Leonard, *Am. Rev. Tuberc.*, 1946, **54**, 287.

6 Youmans, Guy P., Raleigh, Gordon W., and Youmans, Anne S., *J. Bact.*, 1947, **54**, 409.

7 Youmans, Guy P., and McCarter, John C., *Am. Rev. Tuberc.*, 1946, **52**, 432.

TABLE I.
Bacteriostatic Effect of Chloromycetin on 20 Strains of Tubercle Bacilli.

Strain No.	Type	Concentration in μg per ml which completely inhibited growth	
		Without serum	With serum
H37Rv	Human	12.5	12.5
H37RvR*	"	12.5	12.5
1	"	12.5	12.5
1R	"	12.5	25.0
11	"	12.5	12.5
12	"	12.5	12.5
15	"	3.12	12.5
15R	"	3.12	12.5
18	"	1.56	12.5
21	"	6.25	12.5
24	"	12.5	12.5
24R	"	6.25	25.0
69	"	12.5	12.5
69R	"	12.5	12.5
97	"	6.25	12.5
97R	"	6.25	12.5
100	"	12.5	12.5
100R	"	12.5	12.5
111	"	12.5	12.5
48	Bovine	No growth	25.0

* R indicates a streptomycin-resistant strain.

between 6.25 and 12.5 μg per ml to completely inhibit growth. The bacteriostatic activity of chloromycetin for these same 12 strains in the presence of serum was not significantly reduced. The remaining 7 strains which, in the absence of serum were inhibited in their growth to a greater degree by chloromycetin, were, in the presence of serum, as resistant as the other 12. Whether the decrease in sensitivity to chloromycetin shown by the above 7 strains in the presence of serum was due to the inactivation of chloromycetin by serum or was merely a reflection of the stimulation of growth of tubercle bacilli which occurs in the presence of serum is not discernible from these data. While the figures given in Table I represent the least amount of chloromycetin which under the conditions of the test completely inhibited growth, partial retardation of growth was usually noted in one-half or one-fourth this concentration. In any event, chloromycetin *in vitro* is considerably less bacteriostatic for virulent human type tubercle bacilli than is either streptomycin or para-aminosalicylic acid since with these latter substances most strains of tubercle bacilli are completely inhibited in their growth by less than one or 2 μg per ml.[7,8]

TABLE II.
Bacteriostatic Action of Chloromycetin in Combination with PAS and Streptomycin.

	Conc. in μg per ml which completely inhibited growth
Chloromycetin	12.5
PAS	0.78
Streptomycin	0.78
Chloromycetin + PAS	1.56
" + Streptomycin	1.56
" + PAS + Streptomycin	1.56

Since the bacteriostatic activity of many chemotherapeutic agents such as the sulfonamides, sulfones and PAS is markedly affected by the number of organisms used in the test, the bacteriostatic action of chloromycetin was tested using larger numbers of tubercle bacilli. Since a 10-fold increase in the amount of inoculum used in the test resulted in only a 4-fold decrease in bacteriostatic activity, the action of the drug, in contrast to para-aminosalicylic acid[7] is apparently not appreciably influenced by the number of organisms present.

In spite of the relatively low tuberculostatic action of chloromycetin, it was thought advisable to determine *in vitro* the effect of combining this substance with para-aminosalicylic acid and streptomycin. Table II shows the results of these tests. It is apparent that chloromycetin contributed nothing to the bacteriostatic action of such combinations since in no case was the effect greater than that obtained with either para-aminosalicylic or streptomycin alone. There was in fact consistently a slight reduction in bacteriostatic action when chloromycetin was included in the medium. Since this difference is well within the experimental error of the method, no great significance at present can be attached to it.

The initial experiments with chloromycetin to determine its therapeutic effectiveness *in vivo* were conducted with intravenously tuberculized mice by treating subcutaneous-

[8] Raleigh, Gordon W., and Youmans, Guy P., *J. Inf. Dis.*, 1948, in press.

TABLE III.

Results of the Mice Infected with *M. tuberculosis* (H37Rv) and Treated with Chloromycetin in the Diet.

Compound	% of compound in diet	No. mice	% mortality	Avg wt loss or gain in g	Amt gross pulmonary tuberculosis	Type of lesion
Chloromycetin	0.5	20	0.0	—4.1	3.5+	P & NE
	0.25	20	30.0	—3.3	3.84+	P & NE
	0.125	20	65.0	—3.85	3.85+	NE
Para-amino-salicylic acid	1.0	20	0.0	+1.6	2.5+	P
Controls		20	75.0	—4.8	4.0+	NE

1+ — 0-10% involvement of lung with tuberculosis
2+ — 10-25% " " " " "
3+ — 25-50% " " " " "
4+ — 50-100% " " " " "
P — Proliferative lesions
NE — Necrotic-exudative lesions

ly with 5 mg of chloromycetin per day dissolved in 20% propylene glycol. This was divided in 2 daily doses of 2.5 mg each given approximately 8 hours apart. This total daily dosage was selected on the basis of the known toxicity of chloromycetin administered by this route[3] and approached closely the maximal tolerated dose. These experiments failed to demonstrate any suppressive effect on the tuberculous process. Furthermore, marked reaction developed at the site of injection with swelling, edema and eventually ulceration.

When it became apparent that chloromycetin was well absorbed from the gastro-intestinal tract, experiments with intravenously tubercularized mice were conducted in which the compound was administered with the diet. Table III details the results of a typical experiment. Included in this table in addition to the untreated controls is a group of animals treated with 1.0% para-amino-salicylic acid. From the data given in the table, it is readily apparent that there was a relationship between the per cent mortality of the treated mice and the concentration of chloromycetin administered. Those mice which received 0.5% chloromycetin in the diet survived the 21-day period of treatment. The group which received 0.25% showed 30% mortality and the group which received 0.125%, a 65% mortality. The last figure is very similar to the mortality figure for the untreated control group. There was no significant difference between the amount of weight lost by any of the chloromycetin treated animals and the untreated controls; in contrast, the para-aminosalicylic acid treated animals showed a slight increase in average weight. The data given for the amount of gross pulmonary tuberculosis demonstrable at the end of the experimental period in the various groups correlates with the weight response. On the basis of these findings one might question the significance of the mortality figures for the groups of animals receiving the 0.5% and the 0.25% chloromycetin. However, upon microscopic examination, whereas the total amount of involvement of lung substance was large, those animals treated with 0.5% and 0.25% chloromycetin showed a significantly higher proportion of proliferative type lesions as compared with the controls. These findings, as has previously been determined,[8] indicate that the treated animals had a slower evolution of the disease process. The histopathological findings in the mice which received the smallest amount of chloromycetin (0.125%) did not differ from the untreated controls. It can be stated with some confidence that chloromycetin administered orally to intravenously tubercularized mice in a concentration of 0.5%, and possibly 0.25%, exerted a slight suppressive effect upon the disease process. Before the animals which received 0.5% were sacrificed at the termination of the experiment they were bled by cardiac puncture approximately 2 hours after being fed and the small blood samples obtained were pooled. The serum thus obtained was tested for its content of chloromycetin‡ and was found to be 7.0 μg per ml.

‡ Performed by Dwight A. Joslyn, Parke, Davis & Company, Detroit, Mich.

If this one determination represented the average serum concentration of chloromycetin during the course of treatment one would expect the slight borderline therapeutic effect which was noted since this concentration of chloromycetin, while it does not completely inhibit the growth of H37Rv strain *in vitro*, will partially retard the multiplication of the bacilli.

Summary and Conclusions. The antibiotic chloromycetin has been found to be only moderately bacteriostatic for virulent human type tubercle bacilli *in vitro* as compared with streptomycin or para-aminosalicylic acid. The majority of 19 human type strains studied were completely inhibited in their growth by between 6.25 and 12.5 micrograms or more of the drug in the presence of serum. One bovine strain was equally sensitive. This degree of bacteriostatic activity was not markedly affected by the number of tubercle bacilli present nor was the bacteriostatic activity of para-aminosalicylic acid or streptomycin enhanced by the addition of chloromycetin.

When administered subcutaneously, chloromycetin has been shown to be ineffective, whereas, when admixed with the diet in concentrations of 0.5 and 0.25%, it was slightly effective for the suppression of experimental murine tuberculosis.

16330

Subtenolin. An Antibiotic from *Bacillus subtilis*. I. Bacteriologic Properties.

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(Introduced by Henry Tauber.)

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In the course of a search for antibiotic agents in this laboratory, an organism was isolated in February, 1945, that produced a substance showing antibiotic activity for certain gram-positive and gram-negative bacteria. The organism was isolated from nutrient agar plates that had been seeded with *Escherichia coli* and *Staphylococcus aureus*, respectively, and inoculated with material from dusty surfaces in the laboratory. It was identified as *Bacillus subtilis*.* Because of its microbic source and strong enolic properties[1] the name, subtenolin, has been given to the antagonist. The bacteriologic properties of the antibiotic are the subject of this report; its isolation from the harvest and its chemical properties are described in the succeeding report.[1]

Production of Subtenolin. Mediums containing peptones and other complex organic enrichments were found to be very poor for the production of subtenolin. A medium containing *dl*-alanine produced the highest yields and least destruction. With subtenolin, as with subtilin,[2] strong stimulatory action by manganese was observed. Production was slightly stimulated by copper.

The following medium was adopted for use in the production of subtenolin:

* Acknowledgment is made to N. R. Smith of the U. S. Department of Agriculture, Beltsville, Maryland, for the final identification of the organism.

1 Howell, S. F., and Tauber, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 432.

2 Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.*, 1944, **4**, 297.

	%
dl-alanine	0.3
Potassium dihydrogen phosphate	0.5
Magnesium citrate · $14H_2O$	0.25
Magnesium sulfate · $7H_2O$	0.05
Glycerol	2.0
Manganous sulfate · $5H_2O$	0.0004
Cupric sulfate · $5H_2O$	0.00005
Water, distilled, to volume	

The medium was adjusted to pH 6.8-7.1, dispensed 300 ml per one-gallon bottle, and autoclaved at 15 lb for 15 minutes. The bottles were inoculated with 0.5 ml of a standardized spore suspension (tube No. 0.5 McFarland barium sulfate standard) that had been prepared from discrete colonies. To obtain maximum surface exposure, the bottles were incubated in a horizontal position at 36°C for 3 days. Subtenolin activity reached its peak when growth was greatest (3 to 4 days) and remained constant until one or 2 days later when antibiotic activity steadily decreased with increased alkalinity of the culture medium.

Lyophilization of the spores in 25% gelatin proved to be the best method for maintaining the strain of *Bacillus subtilis* for consistent maximum production of subtenolin. By preparing a new batch of lyophilized spores at least every 3 months, the level of subtenolin production was held fairly constant throughout the period of this work.

In practice, the inoculum was prepared by reconstituting the contents of an ampoule of lyophilized spores and preparing tenfold dilutions in saline for plating on FDA agar.[3] After incubation for 4 to 7 days at 30°C, individual rough colonies were selected and suspended in 0.85% NaCl solution. This suspension was standardized and inoculated in the medium described above. Harvesting of the culture was done by screening through several layers of gauze and cotton to remove most of the bacterial pellicle.

General Properties of Subtenolin. Subtenolin diffuses very readily through agar, producing large and clearly defined zones with *Staphylococcus aureus* as the test organism. Many preparations showed the presence of isolated, resistant colonies within the clear area of inhibition.

The antibacterial activity of subtenolin is variously reduced by complex organic substances. In whole, defibrinated rabbit blood there is almost complete loss of activity in 2 hours. Several peptones also reduced the antibacterial effect of subtenolin. However, rabbit or horse serum, FDA broth,[3] and heart infusion broth had no effect when tested by the agar streak method.

Toxicity studies by intraperitoneal injection of mice weighing 17 to 23 g showed the LD_{50} to be 30 to 60 mg (1000 units/mg). Considerable subtenolin activity was found in the urine 15 minutes after injection and, depending upon the dosage, continued 4 to 10 hours. With a dose of 50 to 60 mg (1000 units/mg), subtenolin continued to be excreted for 10 hours after intraperitoneal injection. On the average, 30 to 50% of the administered dose was found in the urine.

Assay Methods and Antibacterial Spectrum. The serial dilution method using FDA broth[3] was the procedure adopted for subtenolin assay. Since there was reduction of subtenolin activity after overnight incubation but none after 5 hours, the tests were read at 5 hours and again at 22 hours. The endpoint selected was the highest dilution of a standard subtenolin solution showing complete inhibition of the test organism. For the more fastidious organisms, heart infusion broth containing 10% rabbit serum and 1% dextrose was used. This medium reduced subtenolin activity 50 to 80% after overnight incubation although no reduction in activity was observed after 5 hours incubation.

The antibacterial spectrum of subtenolin is summarized in Table I. The most susceptible organisms were: *Staphylococcus aureus, Staphylococcus albus, Eberthella typhosa, Escherichia coli, Salmonella enteritidis, Micrococcus conglomeratus,* and *Salmonella schottmuelleri.* Two strains of *Pasteurella* showed marked susceptibility to the antibiotic upon overnight incubation. The growth of *Mycobacterium tuberculosis,* H37Rv in Dubos medium[4] was partially inhibited by a concentration of 2000 units/ml of subtenolin.

Differentiation of Subtenolin from Other Antagonists. Antibiotic substances produced

[3] *Federal Register,* Oct. 17, 1946, **11**, 12128.

TABLE I.
The Antibacterial Spectrum of Subtenolin.

	Minimal inhibiting quantity (mg)	
	5 hr	22 hr
Preparation No. 165, 300 units/mg*		
1. *Staphylococcus aureus*, FDA 209P	.004	0.55
2. " " MB 111	.004	n.i.†
3. " " 30393	.004	n.i.
4. " " H	.0065	n.i.
5. " *albus*, 30389	.0023	0.47
6. *Streptococcus*, sp. (enterococcus group VD)	.023	1.25
7. *Bacillus anthracis*, ATCC 8705	2.0	n.i.
8. *Micrococcus conglomeratus*, MB 78	.024	n.i.
9. *Escherichia coli*, ATCC 7011	.014	1.67
10. *Salmonella enteritidis*	.015	n.i.
11. *Eberthella typhosa*, MB 59	.019	1.0
12. *Salmonella schottmuelleri*, M	.047	2.0
13. " *typhimurium*, ATCC 9148	.078	n.i.
14. *Serratia marcescens*, D	.16	n.i.
15. *Aerobacter aerogenes*, ATCC 8308	.16	n.i.
16. *Klebsiella pneumoniae*, ATCC 9997	.71	n.i.
17. *Pasteurella pestis*, A 1122	i.g.‡	0.055
Preparation No. 176, 550 units/mg*		
1. *Staphylococcus aureus*, FDA 209P	.0021	0.28
2. *Streptococcus pyogenes*, C203	.54	2.7
3. *Diplococcus pneumoniae*, type III	2.72	n.i.
4. *Clostridium novyi*, AMS	i.g.	0.55
5. " *perfringens*, AMS	i.g.	0.55
6. " *fallax*, AMS	i.g.	0.91
7. " *tertium*, AMS	i.g.	0.91
8. " *histolyticum*, AMS	i.g.	1.1
9. " *oedematis maligni*, AMS	i.g.	1.4
10. *Bacillus subtilis* (subtenolin producing strain)	n.i.	n.i.
11. " " R	n.i.	n.i.
12. *Micrococcus lysodeikticus*, MB 79	.17	n.i.
13. *Pasteurella*, sp., MB 90	i.g.	0.041
14. *Eberthella typhosa*, PCI 412	.0065	0.303
15. *Escherichia coli*, P6	.018	0.68
16. " " MB 60	.064	2.7
17. " " VD	.026	n.i.
18. *Pseudomonas aeruginosa*	2.7	n.i.
19. *Neisseria gonorrhoeae*, strain 1	i.g.	1.1
20. " " strain 2	i.g.	1.1
Preparation No. 189, 850 units/ mg*		
1. *Staphylococcus aureus*, FDA 209P	.0014	0.17
2. *Brucella abortus*, USDA 2610	i.g.	n.i.
3. " *melitensis*, USDA K1957	i.g.	n.i.
4. " *suis*, USDA, 8452	i.g.	n.i.
5. *Salmonella paratyphi*, ATCC 9150	n.i.	n.i

* One unit is defined as the minimal concentration of subtenolin completely inhibiting the growth of *Staphylococcus aureus*, FDA 209P in total volume of one ml after five hours incubation period. In the determination of the antibacterial spectrum, a reference solution was prepared by dissolving subtenolin in distilled water to give a concentration of 15,000 units/ml.

† n.i. = no inhibition.

‡ i.g. = insufficient growth.

by strains of *Bacillus subtilis* include subtilin,[2] bacitracin,[5] bacillin,[6] eumycin,[7] and subtilysin, a lytic principle obtained by Vallée[8] from culture filtrates. In addition, coli-

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[5] Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

[6] Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1946, **51**, 363.

[7] Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

[8] Vallée, M., *C. R. Soc. biol.*, 1945, **139**, 148.

statin, apparently produced by an unidentified member of the genus *Bacillus,* has been reported.[9] Subtenolin differs from these antagonists of similar origin as described below.

Subtilin. Subtilin is extracted from the cellular material of the pellicle and is light-sensitive. Subtenolin is extracted from the culture medium and is not light-sensitive.

Bacitracin. Bacitracin does not inhibit *Escherichia coli, Aerobacter aerogenes* nor *Eberthella typhosa,* but these organisms are susceptible to the action of subtenolin. Unlike bacitracin, subtenolin is relatively inactive against *Streptococcus pyogenes,* pneumococcus and clostridia.

Bacillin. Subtenolin and bacillin display a loss of activity in the presence of certain complex organic substances (although not to the same degree), and their production is stimulated by manganese. On the other hand, carbohydrates promote the production of bacillin but interfere with the production of subtenolin. Also, carbohydrates are required for demonstrating the anticoli activity of bacillin but not of subtenolin. Bacillin is not excreted in the urine of mice† but subtenolin activity has been found in the urine of mice after intraperitoneal injections. *Streptococcus pyogenes* and *Diplococcus pneumoniae* Type III are relatively susceptible to bacillin but resistant to the action of subtenolin. In comparative studies with bacillin, subtenolin was found to be 5 times as active against *Staphylococcus aureus* 209 P on FDA agar.

Eumycin. Eumycin was reported to have no activity against *Eberthella typhosa* and colon bacilli and only slight activity against staphylococci. Subtenolin definitely inhibited these organisms, especially staphylococci.

Subtilysin. Subtilysin shows activity for various clostridia and none for staphylococci. Subtenolin shows the opposites of these activities.

Colistatin. The presence of glucose in the medium is required for colistatin production, but this sugar is not favorable for subtenolin production. Contrary to subtenolin, colistatin inhibits the pneumococcus.

Summary and Conclusions. 1. An antibiotic, tentatively designated subtenolin, was isolated from a strain of *Bacillus subtilis.*

2. Although the antagonist displays its greatest activity against staphylococci, the growth of certain gram-positive as well as gram-negative organisms is inhibited.

3. The growth of *Mycobacterium* tuberculosis is partially inhibited by subtenolin.

4. Subtenolin activity has been detected in urines recovered from intraperitoneally inoculated mice.

5. Acute toxicity studies show that the LD_{50} of subtenolin for mice inoculated intraperitoneally is 30 to 60 mg (30,000-60,000 units).

[9] Gause, G. F., *Science,* 1946, **104**, 289.

† Personal communication from H. B. Woodruff of Merck and Company.

16331

Subtenolin. An Antibiotic from *Bacillus subtilis.* II. Isolation and Chemical Properties.

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The method of isolation and the chemical properties of subtenolin, an antibiotic produced by a strain of *Bacillus subtilis,* are described in this paper. The method used in the isolation of the antibiotic is based on its ability to be concentrated by adsorption on activated carbon, its solubility in methyl alcohol, its insolubility in butanone, and its

stability when dried from a methyl alcohol solution. Subtenolin has very interesting chemical properties. It is extremely sensitive to drying in the presence of water. However, in aqueous solution, it is very stable to heat. This antibiotic has strong enolic properties. The bacteriological study of the antibiotic is described in the preceding paper.[1]

Experimental. Isolation of the Antibiotic. Ten liter portions of the medium, the composition of which has been given in the preceding article,[1] were processed each time. The pH of the broth was usually between 6.7 and 6.8. The pellicles were removed by screening through several layers of cheesecloth, then by centrifuging in a Sharples supercentrifuge. Forty grams of charcoal (Darco G-60) were added per liter of culture fluid. The mixture was shaken during a period of 20 minutes and filtered on Buchner funnels. The carbon cake was washed with 2 liters of water and transferred to a 5-liter bottle. The antibiotic was eluted with 2 liters of methyl alcohol by shaking for 30 minutes. After filtration the eluate was concentrated *in vacuo* at 40°C to 30 ml.

The concentrated eluate was poured into 1500 ml of butanone (contained in a beaker) and stirred with a glass rod until solidification of the antibiotic took place. The precipitate was washed twice with 100 ml portions of butanone and twice with 100 ml portions of ether. The antibiotic was extracted from the precipitate with one 80 ml and two 30 ml portions of methyl alcohol. The mixture was filtered to remove undissolved material. The white residue on the filter paper was washed with two 30 ml portions of methyl alcohol. Upon solution in water this material showed insignificant activity. The combined methyl alcohol extracts were evaporated to 8 ml *in vacuo* at 45°C. A small amount of inactive material which separated at this point was removed by centrifuging and discarded. The methyl alcohol solution was evaporated to dryness *in vacuo* at 45°C. The dried preparation was stored *in vacuo* over $CaCl_2$.

The yield of subtenolin was about 1.0 g of light yellow powder per 10 liters of medium. Preparations with 1000 to 1600 dilution units per milligram, representing 25 to 50% recovery, were obtained. It is important to note that when solutions of the antibiotic containing more than traces of water are evaporated to dryness, the antibiotic is largely destroyed. Apparently most of the water present in the concentrated eluate was removed by the butanone treatment without inactivating the antibiotic.

Chemical Properties of Subtenolin. The antibiotic is very soluble in water, in ethylene glycol, in 95% methyl alcohol, and in many organic solvents containing a small amount of water. It is insoluble in acetone, butanone, ether, and 95% ethyl alcohol. It is not precipitated by acids or alkalis. Subtenolin is very heat stable. No loss of activity occurs when a solution in water is autoclaved for 15 minutes at 15 lb pressure. No destruction takes place at pH 2.0 during 18 hours at room temperature. The dried preparations may be kept for months in a vacuum without loss of potency. Subtenolin dialyzes readily through cellophane membranes and is somewhat hygroscopic in the dry state. The antibiotic in neutral solution gives a strong "peroxidase" test with p-phenylenediamine and hydrogen peroxide (purple color).[2] It gives a strong enol test,[2] a typical Molisch test, a blue color with ninhydrin, and a dark brown color with ferric chloride. It forms a crystalline hydrazone with 2,4-dinitro-phenylhydrazone in alcoholic solution. When a saturated solution of picric acid in ethyl alcohol is added to a concentrated aqueous solution of subtenolin, the solution turns brown and a crystalline substance is obtained in the form of rosettes. This crystalline material is inactive. Subtenolin reduces permanganate, iodine, and bromine in the cold, and Benedict's qualitative sugar reagent and ammoniacal silver nitrate solution on boiling. It reduces Shaffer-Somogyi's sugar reagent one-half as much as penicillin G and about one-sixth as much as glucose when two milligrams of each of the substances were used

[1] Hirschhorn, H. N., Bucca, M. A., and Thayer, J. D., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 429.

[2] Feigl, F., *Qualitative Analysis by Spot Tests*, Nordemann Publishing Company, Inc., New York, 1939.

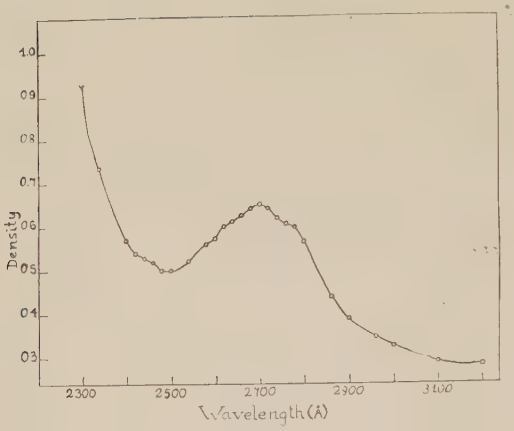

FIG. 1.

Adsorption spectrum of subtenolin in the ultraviolet region. One cc contained 0.4 mg of the antibiotic in distilled water. The pH was 6.52.

per test solution. The purified product contains 51.4% carbon, 7.00% hydrogen, 7.88% nitrogen, and 0.81% sulfur.

A solution of subtenolin in normal hydrochloric acid becomes rose colored upon boiling. Upon the addition of an excess of alkali the solution turns yellow but returns to rose color when reacidified. Subtenolin is destroyed by hydrogen sulfide but not by cysteine. Pepsin (Parke, Davis & Company, 1:3000), trypsin (Difco Laboratories, 1:250), polidase-S (Schwarz Laboratories), and tyrosinase (1830 units per ml)[3] have no effect on the antibiotic.

The antibiotic exhibits a striking absorption of light in the ultraviolet region of the spectrum (see Fig. 1). The curve has a high sharp peak at 2700 A and shows a minimum absorption at 2500 A. The Beckman spectrophotometer was used.

Discussion and Summary. It may be seen from Table I that subtenolin is quite different chemically from other antibiotics such as streptomycin, penicillin G, gramicidin, and bacitracin. Subtenolin, bacitracin, bacillin, subtilin, and eumycin are products of various strains of *B. subtilis*. Gramicidin[4] and bacitracin[5] appear to be peptides. Subtilin, which

TABLE I. Some Chemical Properties of Subtenolin and Other Antibiotics.

		Subtenolin, 1000 u/mg	Penicillin G Sodium (Com'l Solvents Corp. 1622 u/mg)	Streptomycin (Merck & Co. $CaCl_2$ Complex Crystalline)	Gramicidin (Wallerstein Co.)	Bacitracin (Com'l Solvents Corp., 34 u/mg)
Molisch's Test		+	—	—	—	—
Benedict's Qualitative Sugar Test		+	+	—	—	—
Millon's Test		—	—	—	—	+
Hopkins-Cole Test		—	—	Light blue	Intense purple	Purple
Biuret Test		Yellow on boiling	—	,, ,,	Intense violet	,,
Ninhydrin Test		Blue	—	—	Blue	,,
Trichloroacetic acid (10%)		—	—	—	Heavy precipitate	Turbid
Ferric Chloride		Dark brown	White precipitate	Yellow	—	Yellow
Enol Test		+	—	—	—	—
Indicator Test		Rose (turns yellow in alkaline sol.)	—	—	—	—
p-Phenylenediamine Test	In neutral sol.	Dark purple	Same as control	Same as control	Same as control	Same as control
	In acid sol.	Yellow	,, ,, ,,	,, ,, ,,	,, ,, ,,	,, ,, ,,

In all tests 5 mg of the respective antibiotics were used except in the *p*-phenylenediamine test in which 0.5 mg of each of the antibiotics was used. The *p*-phenylenediamine test was carried out by adding 0.5 ml M phosphate buffer pH 7.0, 0.5 ml of 1% *p*-phenylenediamine in water, and 0.5 ml 3% hydrogen peroxide to 0.1 ml of the respective antibiotic solutions. When this test was carried out in acid medium 2 M acetic acid was substituted for the phosphate solution.

[3] Miller, W. H., Mallette, M. F., Roth, L. J., and Dawson, C. R., *J. Am. Chem. Soc.*, 1944, **66**, 514.

[4] Hotchkiss, R. D., *Adv. Enzymol.*, 1944, **4**, 153.

[5] Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

is extracted from the pellicles of a particular strain of *B. subtilis,* is of unknown chemical composition.[6] Eumycin is prepared by acid precipitation of the medium.[7] The chemical relationship between bacillin and subtenolin has not been ascertained since data pertaining to the chemical nature of bacillin is not available for comparison.[8]

Subtenolin has a low molecular weight. Its chemical and physical properties indicate that it contains a resonating double bond, phenolic groups, a very active enolic group, and an aromatic aldehyde radical. This antibiotic gives typical color reactions such as the Molisch and enol reaction. These tests, and the indicator test shown in Table I, may be used in its identification. Although the antibiotic has not been obtained in a pure state, its chemical and antibiotic properties, particularly its stability in aqueous solution or when dry, make it an interesting object for further studies.

[6] Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., *Arch. Biochem.*, 1947. **15**, 1.

[7] Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

[8] Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1946, **51**, 371.

16332

Respiratory Arrest in Rabbits Exposed to Hypoxia after Dibenamine.*

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The observations here reported were made as part of a systematic elucidation of the effect of alteration of function of the autonomic nervous system on the response of the intact animal to hypoxia.

Rabbits underwent early and sudden respiratory arrest on exposure to hypoxia after receiving Dibenamine (N, N-dibenzyl-β-chloroethylamine) in ethyl alcohol and propylene glycol aa by vein. Control animals underwent such arrest very rarely even under more prolonged similar exposure to hypoxia.

Procedure. Normal rabbits (weight 1.5 to 4 kg) under intravenous pentothal anesthesia were subjected to tracheotomy. Procaine hydrochloride 2% was infiltrated through the margins of the incision and skin closure around the cannula was made with skin clips.

The rabbits were restrained in the supine, head low position on a table offering a slope of 15° from the horizontal in order to aid the venous return. After recovery (minimum 30 min.) from the general anesthesia the inspiratory ventilation volume on room air was measured by spirometer. This was made possible by an hydraulic flutter valve‡ interposed between the tracheal cannula and the spirometer. The dead space of the system was 3 to 5 cc. This compares favorably with the dead space of the eliminated respiratory tract above the cannula in the rabbit.

Measurements were then continued as the animal was abruptly shifted to an atmosphere of 5% oxygen in helium for a period of 5 minutes. After one or a series of such control

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† Work done in partial fulfillment of the requirements for the degree of Master of Arts, Boston University Graduate School.

‡ A modification of the type pictured on page 206, Jackson, D. E., *Experimental Pharmacology and Materia Medica,* 2nd Edition, 1939, C. V. Mosby Co., St. Louis, Mo.

TABLE I.
Occurrence of Respiratory Arrest on Exposure to *Circa* 5% O_2 for 5 Minutes.

Treatment	No. of rabbits showing no arrest	No. of rabbits showing arrest
Normal	49	2
Dibenamine in Propylene Glycol and Ethyl Alcohol āā 12-24 mg/kg*	2	20
Dibenamine in 50% Alcohol 12-24 mg/kg	1	4
Dibenamine in 50% Propylene Glycol 12-24 mg/kg	1	5
Propylene Glycol 50% 2 cc	6	0
Alcohol 50% average 0.5 cc	6	0
Propylene Glycol and Absolute Alcohol āā average 0.5 cc	6	0

* By Chi square test the probability of chance distribution of 29 positive in 33 as opposed to 2 positive in 49 is less than 1 in 10,000.

observations on the effect of hypoxia, Dibenamine (at a concentration of 50 mg/cc in the chosen medium)§ was administered in marginal vein of ear in calculated dose over a period of one minute. Usually the primary dosage of Dibenamine was 12 mg/kg.

After a variable delay the ventilation volume on room air was measured and the animal was again presented with the hypoxic atmosphere for 5 minutes. If respiratory arrest occurred artificial ventilation was established after 1 to 2 minutes of apnea, by means of human expired air through the tracheal cannula. Successive periods of hypoxia were rarely at intervals less than 20 minutes.

Gas mixtures were made in large rubber bag of some 1500 liter capacity and analyzed for oxygen content repeatedly through the day's experiments. Maximum variation of different days' mixtures was 4.0-5.5% oxygen (mean 5%±0.5).

Observations. Table I records the incidence of respiratory arrest in rabbits exposed to hypoxia for 5-minute periods after various medications.

Normal Controls. The 2 cases of respiratory arrest in 49 normals showed arrest only once each in several trials. Time required for arrest to occur was 230 and 240 seconds respectively. It was repeatedly shown that 5-minute periods of hypoxia did not predispose to respiratory arrest in later 5-minute periods of exposure (11 animals up to 9 trials each over periods up to 6 hours).

Dibenamine: Incidence. In the total series of 38 animals given Dibenamine 5 died within 10 minutes of the intravenous injection of 12 mg/kg. Twenty-three animals showed arrest during hypoxia after the first 12 mg of the drug. Six animals required a second dose at the end of an hour or more before arrest would result from hypoxia. The remaining 4 animals never showed respiratory arrest.

The pattern of respiratory arrest after Dibenamine was bizarre. The rise of ventilation volume at the beginning of the period of hypoxia (average 59% rise) differed little from that of the control hypoxic condition (average 72% rise). However, after an average exposure of 158 seconds (in 44 trials, range 30 to 300 seconds) the respiration became more labored, followed abruptly by an apparently tonic contraction of the diaphragm, accompanied by twitching movements of the abdomen. Simultaneously the tidal volume fell to zero and the tonic contraction faded out. Thereafter no respiratory movements appeared in most animals though one or two gave a feeble gasp reflex.

Duration of susceptibility to respiratory arrest. In animals tested serially to determine the time course of susceptibility to respiratory arrest on exposure to hypoxia, arrest could be elicited at the earliest in most animals between 20 and 40 minutes (mean 33 minutes) after the administration of the drug. Susceptibility endured throughout the period of test (up to 4 hours) in a number of animals. In others the ability to compensate for hypoxia returned, after as little as

§ Media used included Propylene glycol and absolute alcohol āā most commonly, Propylene glycol and water āā or absolute alcohol and water āā on other occasions.

48 minutes following administration of Dibenamine.

Controls receiving solvents without Dibenamine. See Table I.

Discussion. That the susceptibility to respiratory arrest is not dependent on some residual effect of the pentothal was demonstrated by the occurrence of respiratory arrest in cats under the same conditions except that cyclopropane was utilized during the preparatory period. The pilot experiments in the cat also showed that the susceptibility to arrest is not species specific in the rabbit alone.

That the helium was not an active factor in the elicitation of respiratory arrest was shown in animals tested on nitrogen and oxygen (5%). Five minutes on this mixture never caused arrest in several trials in each of 11 animals tested before Dibenamine. It was at least as potent in producing respiratory arrest after Dibenamine.

That the phenomenon observed was a true respiratory arrest was shown by the demise of 8 animals allowed to go untreated in the apnea which occurred during hypoxia after Dibenamine. No animal ever recovered spontaneously from this state of arrest. On resuscitation spontaneous respiration was reestablished promptly after a minute or more of artificial ventilation with human expired air.

As to the mechanism of action of Dibenamine in inducing respiratory arrest during hypoxia little evidence is available. Pilot experiments have shown that bilateral vagotomy, with its removal of inhibitory sensory discharge does not prevent the occurrence of arrest on hypoxia after Dibenamine (3 animals). Three experiments suggested that Dibenamine does reverse the normal pressor response to hypoxia in rabbits as in other species. However, the fall of blood pressure is inconstant and respiratory arrest has been noted unaccompanied by a depressor response. Acapnia seems an unlikely cause of the apnea, since the hyperventilation normally seen in hypoxia of this grade is at least as great as that after Dibenamine. Finally, a direct depressant action of Dibenamine on the respiratory center seems unlikely, since the center still responds to the carotid body under hypoxia by inducing hyperventilation.

Summary. Rabbits given Dibenamine 12 to 24 mg/kg in propylene glycol or ethyl alcohol by vein show sudden respiratory arrest within 3 minutes of exposure to hypoxia (circa 5% O_2 in helium).

16333

Results of Heterophile Antibody Agglutination and Kahn Tests in Patients with Viral Hepatitis.*

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Attempts to develop a *specific* immunologic test for viral hepatitis have been unsuccessful, up to the present, although positive results have been described in certain serologic reactions in this disease.[1,2,3] Eaton *et al.*[2] reported that 34% of a group of pa-

* This work was conducted under the auspices of the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

1 Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putnam, P., and Schwentker, F. F., *Am. J. Hyg.*, 1944, **39**, 337; Am. J. Hyg., 1944, **40**, 35.

tients in the acute phase of hepatitis developed an heterophile antibody which agglutinates sheep erythrocytes in a titer of 1:40 or greater. The titer of this antibody rarely rises above 1:160, and may be differentiated from that appearing in patients with infectious mononucleosis by its absorbability on boiled guinea pig kidney and human liver.

It has also been reported that a certain number of patients with viral hepatitis develop transient falsely positive tests for syphilis,[4] and as many as 20% of one group of patients were reported to have falsely positive Kahn and Wassermann tests during the course of acute hepatitis.[5]

Such a substantial percentage of positive serologic tests, occurring during the course of hepatitis, might constitute a problem in diagnosis. Certain similarities between hepatitis and infectious mononucleosis, such as the presence of cervical lymphadenopathy and lymphocytosis as well as the frequent occurrence of evidence of hepatic dysfunction in the latter disease, make differential diagnosis more difficult.

During recent years, experiments conducted under the auspices of the Army Epidemiological Board on the transmission of infectious hepatitis to volunteers have made it possible to study certain serologic reactions in these patients. In addition, a recent opportunity to survey the serologic response of a large number of patients with hepatitis in an American Army hospital has provided certain data. It is the purpose of this paper to report on the results of the heterophile antibody agglutination, Kahn, and cold agglutinin tests in these two groups of patients.

Method and Materials. Subjects. The patients are divided into 2 groups. *Group I:* 30 previously healthy male volunteers, ranging in age from 19 to 29 years, contracted infectious hepatitis experimentally, following inoculation or ingestion of material known to contain infectious hepatitis virus. The strain of virus used in these experiments has been previously described.[6] *Group II:* 478 subjects were patients in an Army hospital in Germany from June 1947 to February 1948. They had contracted acute hepatitis during the performance of duty. It is quite probable that many of these cases were examples of homologous serum jaundice, but in view of the fact that there are no means at present for differentiating infectious hepatitis from serum hepatitis clinically, the cases have all been classified as viral hepatitis. The diagnosis of hepatitis was made in both groups on the basis of characteristic symptoms and signs, accompanied by consistent deflection of appropriate tests of hepatic function. All patients in this report had clinical jaundice.

In Group I, the heterophile antibody agglutination, Kahn, and cold agglutinin tests were determined before experimental inoculation. All were negative. These subjects were tested again during the 2nd, 3rd and 4th weeks of disease, and sera with positive reactions were retested at weekly intervals until negative.

In Group II, these tests were performed in various stages of infectious hepatitis, ranging from the 1st to the 30th week. Numerous serial determinations were made, and the majority of tests were done during the first 8 weeks of disease. Sera with positive reactions were retested at weekly intervals until negative.

Results. Heterophile antibody agglutination tests. Technique of test. The tests were carried out according to the method of Stuart.[7] Sera with a titer of 1:56 were absorbed on boiled guinea pig kidney and retested.

The results of the determinations in 508 patients represented in Groups I and II are

2 Eaton, M. D., Murphy, W. D., and Hanford, V. L., *J. Exp. Med.*, 1944, **79**, 539.

3 Olitski, L., and Bernkopf, H., *J. Infect. Dis.*, 1945, **77**, 60.

4 Waelsch, J. H., *Brit. Med. J.*, 1946, March 9, 353.

5 Kuzell, W. C., and Puccinelli, V., *Bull. U. S. Army Med. Dept.*, 1944, **80** (Sept.), 3.

6 Havens, W. P., Jr., Ward, R., Drill, V. A., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 206.

7 Stuart, C. A., Infectious Mononucleosis (in) *Diagnostic Procedures and Reagents*, 2nd Ed., New York, Am. Pub. Health Assn., 1945, p. 449.

TABLE I.
Results of Heterophile Antibody Agglutination Tests in 508 Patients with Viral Hepatitis.

Week of disease	No. of patients Tested	Positive* 1:56
1	11	0
2	116	1
3	167	2
4	192	4
5	158	3
6	153	0
7	132	1
8-12	372	3
13-17	75	1
18-30	37	1

* After absorption on boiled guinea pig kidney, titer was 1:7 or negative. The first positive test for each patient is recorded. Subsequent serial determinations of positive tests are not included in this table.

TABLE II.
Results of Kahn Tests in 388 Known Non-luetic Patients with Viral Hepatitis.

Week of disease	No. of patients Tested	Positive*	Doubtful*	Week tests became negative
1	70	3	1	2nd, 3rd 14th
2	175	2	4	3rd, 5th, 7th
3	237	0	3	4th, 6th, 7th
4	247	0	1	5th
5	212	1	0	14th
6	195	0	1	7th
7	153	0	0	
8-12	290	0	0	
13-17	58	0	0	
18-30	8	0	0	

* All tests reported as positive or doubtful had less than 10 Kahn units. The first positive test for each patient is recorded. Subsequent serial determinations of positive tests are not included in this table.

recorded in Table I. The volunteers in Group I, who were all known to have negative tests before experimental inoculation with hepatitis virus, failed to develop positive reactions during the 2nd, 3rd or 4th week after onset of disease. In Group II, none of the patients had titers of heterophile antibody above 1:14 at the 2-hour reading, although 41 patients had titers of 1:28 at the 12-hour reading. Sixteen patients had positive tests at the 12-hour reading with titers of 1:56 which were reduced to 1:7 or negative following absorption on boiled guinea pig kidney.

Kahn tests. Technique of test. The standard and quantitative Kahn tests were carried out according to methods recommended by the U. S. Army.[8]

The results of testing 388 patients in Groups I and II are recorded in Table II. One of the volunteers in Group I developed a positive Kahn test in the 2nd week of disease which became negative in the 7th week. It is of interest to note that this patient had a superimposed bacteremia with *S. cholera suis*.[9] In Group II, 358 patients, who were known not to have syphilis, were tested. Six patients developed positive Kahn tests, and 10 had doubtfully positive tests. These tests became negative between the 3rd and 14th week of disease in all patients.

Cold agglutinin tests. Technique of tests. The tests were carried out according to the method recommended by Bray.[10]

The results of testing 323 patients in Groups I and II are recorded in Table III.

TABLE III.
Results of Cold Agglutinin Tests in 323 Patients with Viral Hepatitis.

Week of disease	No. of patients Tested	Positive* 1:32	Week test became negative
1	37	0	
2	81	0	
3	106	0	
4	108	0	
5	79	0	
6	79	0	
7	60	1	8th
8	45	1	9th
9-12	111	0	
13-16	40	0	
17-23	16	0	

*Tests reverted to negative after standing 3 hours at room temperature. The first positive test for each patient is recorded. Subsequent serial determinations of positive tests are not included in this table.

[8] Turner, T. B., and Rein, C. R., Sero-diagnosis of Syphilis, Chap. XII, (in) Simmons and Gentzkow's *Laboratory Methods of the United States Army*, 5th Ed., Philadelphia, Lea and Febiger, 1944, p. 124.

[9] Havens, W. P., Jr., and Wenner, H. A., *J. Clin. Invest.*, 1946, **25**, 45.

[10] Bray, W. E., *Synopsis of Clinical Laboratory Methods*, 3rd Ed., 1944, St. Louis, C. V. Mosby Company, p. 163.

The volunteers in Group I, who were all known to have negative tests before experimental inoculation with hepatitis virus, failed to develop positive reactions during the 2nd, 3rd or 4th week after onset of disease. In Group II, 2 patients had positive tests with a titer of 1:32, which reverted to negative after standing at room temperature for 3 hours.

Summary. Heterophile antibody agglutination, Kahn, and cold agglutinin tests were performed in 2 groups of patients with viral hepatitis. Sixteen out of 508 patients (3%) developed positive heterophile antibody tests with titers of 1:56 which were reduced to 1:7 or negative by absorption on boiled guinea pig kidney. A rise and/or fall in titer of antibody was demonstrable in serial weekly determinations. Out of 388 patients who were known not to have syphilis, 6 (1.5%) developed positive Kahn tests, and 10 others (2.5%) had doubtful tests. Two out of 323 patients had cold agglutinins present in a titer of 1:32.

The relatively small number of positive heterophile antibody agglutination and Kahn tests is in contrast to reports of others[2,5] who have found a considerably higher percentage of positive tests.

16334

Factors Influencing the Growth of Integumentary Pigment in Fishes. I. The Role of Light.*†

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It is a common observation that light is associated with changes in body pigmentation such as "tanning." Numerous clinical observations[1–5] indicate that certain types of pigmentation deficiencies in man may be corrected by light treatment. In lower mammals Bissonnette and others have demonstrated that pigmentation may be influenced by illumination while experimental evidence has accumulated indicating the importance of light in producing integumentary pigmentation in lower vertebrates. Cunningham[6–8] working with flatfishes; Herbst and Ascher[9] using salamander larvae; Vilter[10] studying axolotls; Sumner and Wells;[11] Sumner and Doudoroff;[12] Odiorne;[13] Sumner;[14-16] Os-

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† Contribution No. 427 of the Woods Hole Oceanographic Institution whose research facilities were generously provided for this investigation.

‡ Now located at Syracuse University.

1 Montgomery, D., *J. Cut. and Urin. Dis.*, 1904, **22**, 17.

2 Buschke, A., *Med. Klinik*, 1907, **33**, 983.

3 Buschke, A., and Mulzer, P., *Berl. Klin. Wchnschr.*, 1907, **44**, 1575.

4 Moser, *Med. Klinik*, 1907, **45**, 1363.

5 With, C., *Brit. J. Dermat.*, 1920, **32**, 145.

6 Cunningham, J. T., *Zool. Anzeiger*, 1891, **14**, 27.

7 Cunningham, J. T., *J. Marine Biol. Assn. United Kingdom*, 1893, **3**, 111.

8 Cunningham, J. T., *J. Marine Biol. Assn.*, 1895, **4**, 53.

9 Herbst, C., and Ascher, F., *Roux Arch. Entw. Mech. Organ*, 1927, **112**, 1.

10 Vilter, V., *C. R. Soc. Biol.*, 1931, **108**, 774.

11 Sumner, F. B., and Wells, N. E., *J. Exp. Zool.*, 1933, **64**, 377.

12 Sumner, F. B., and Doudoroff, P., *Pro. Nat. Acad. Sci.*, 1937, **23**, 211.

13 Odiorne, J. M., *J. Exp. Zool.*, 1937, **76**, 441.

14 Sumner, F. B., *Am. Naturalist*, 1939, **123**, 219.

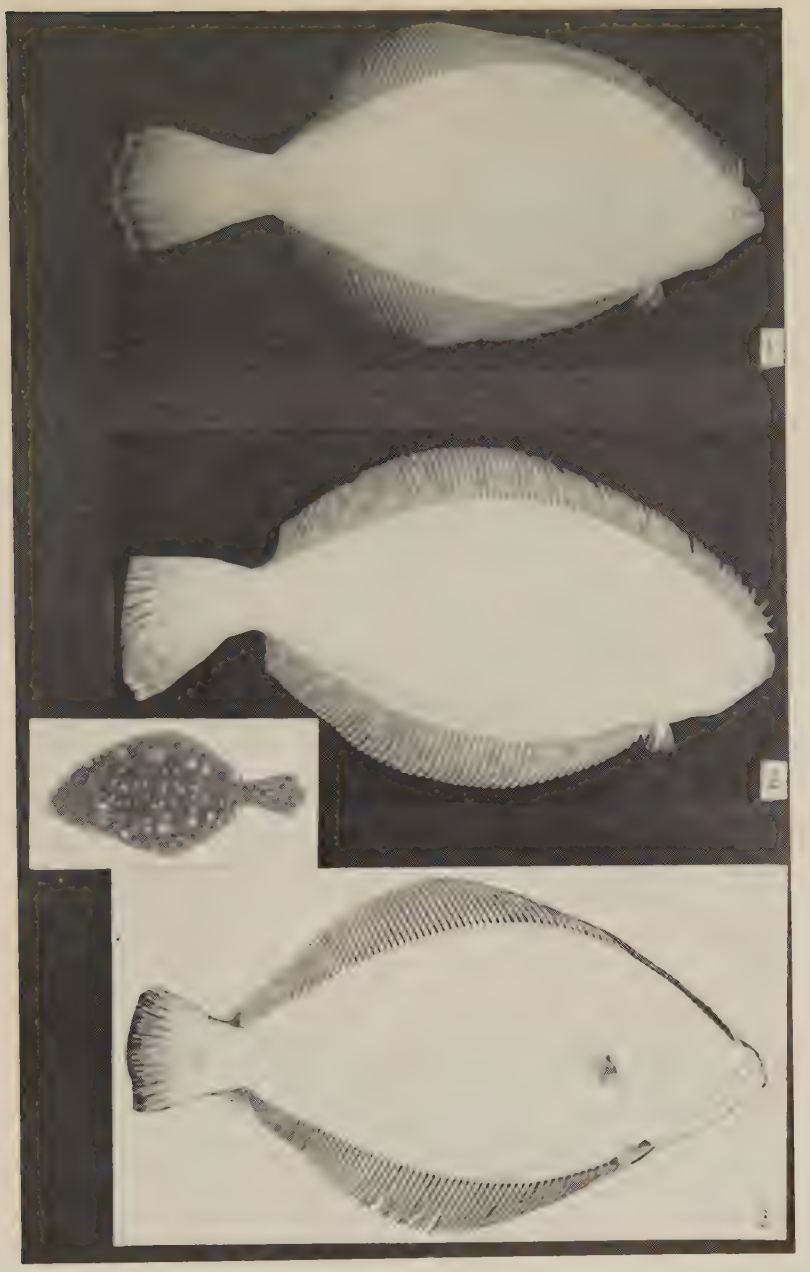

PLATE I.

All figures shown are photographs of the lower surface of living summer flounders. Each animal is selected as typical for the particular experimental situation.

FIG. 1. Normal control freshly caught. The entire ventral surface is free from melanophores.

FIG. 2. Fish black-adapted 2 days, blinded and maintained in darkness 30 days. Entire surface is white as in the control fish except for a few pigmented regenerated scales.

FIG. 3. Animal black-adapted 3 days, blinded and kept for 41 days in a black tank brightly lighted from above. About 2.0 Weston units of light. Somewhat more pigment has developed here than in either of the above figures.

born;[17-21] and Dawes[22] experimenting with various species of fishes have all reported experiments concerning integumentary pigmentation in which light was an important factor. In recent work[17-21] it was found that melanophore development in normally non-melaninated areas depended upon the maintenance of two general conditions. First, the internal environment must be one which favors darkening in the normally pigmented areas; and, second, the external environment must provide illumination (direct or by reflection) to the integument.

The experiments reported here were designed to obtain more detailed information on the role of light in the development of melanophores on the normally unpigmented surface of the summer flounder.

Materials and Methods. Summer flounders freshly caught from Woods Hole waters were used for the experiments. The water temperature for all experiments ranged between 18 and 21°C. The experimental fishes were background-adapted in black tanks illuminated artificially from above. Light intensity (reflected or transmitted) was measured by a Weston exposure meter. The following modifications were made on the ventral illumination tubs previously described:[17] (a) removable false bottoms of perforated glass were rested upon glass supports ¾ inches above the permanent glass floor to allow water to circulate between the 2 glass plates and protect the lower surface of the fishes from overheating; (b) three independent water inlets near the bottom of the tub provided rapid circulation and adequate overflows connected to variable level standpipes allowed easy adjustment of the water level; (c) the light intensity was increased by using a total of 600 Watts for each glass bottomed tub and the walls and ceiling of these special tubs were white to produce a high incidence of internal reflection.

After being black-adapted, the experimental flounders, 106 in all, were totally blinded by enucleation. Opaque masks of black cloth were held in position by suturing each corner to the base of an adjacent fin ray. This kept the mask securely in place without hampering the normal movements of the fishes. Photographic records of the living fishes were made at frequent intervals, supplemented by others of preserved animals.

Adequate unoperated control animals (Fig. 1) in a natural environment were available for purposes of comparison as the experiments progressed.

Experimental. Summer flounders previously black-adapted and totally blinded were placed in total darkness (Exp. 1); subjected to different amounts of overhead illumination where mostly *reflected* light reached the ventral surface (Exp. 2, 3 and 4); exposed to *direct* intense illumination ventrally without masking local areas (Exp. 5) or protecting parts of the ventral surface with opaque masks (Exp. 6).

Exp. 1. Summer flounders black-adapted then blinded as previously described were kept in total darkness from 2 to 6 weeks. Without exception these animals failed to develop any general melanination on the ventral surface (Fig. 2). Here and there a black scale did appear but all were cases of regenerating scales where the original had been lost during experimental manipulation. This will be considered as a constant in subsequent experiments described here. The pigmentation of regenerated scales comprises a special situation which must not confuse the objectives of these experiments.

Exp. 2, 3, 4. These 3 experiments embraced changes in intensity of light source and shade of background so that the light reflected from the surfaces was 0.1 to 0.2, 2.0, and 16.0 Weston Units respectively. Considering the findings from these experiments collectively it was observed that the most ventral melanin appeared on those fishes illuminated most intensely and long-

15 Sumner, F. B., *J. Exp Zool.*, 1940a, **83**, 327.

16 Sumner, F. B., *Biol. Rev. Cambridge Philosophical Soc.*, 1940b, **15**, 351.

17 Osborn, C. M., *Proc. Nat. Acad. Sci.*, 1940a, **26**, 155.

18 Osborn, C. M., *Anat. Rec. Suppl.*, 1940b, **78**, 70.

19 Osborn, C. M., *Anat. Rec. Suppl.*, 1940c, **78**, 167.

20 Osborn, C. M., *Biol. Bull.*, 1941a, **81**, 341.

21 Osborn, C. M., *Biol. Bull.*, 1941b, **81**, 352.

22 Dawes, B., *J. Exp. Biol.*, 1941, **18**, 26.

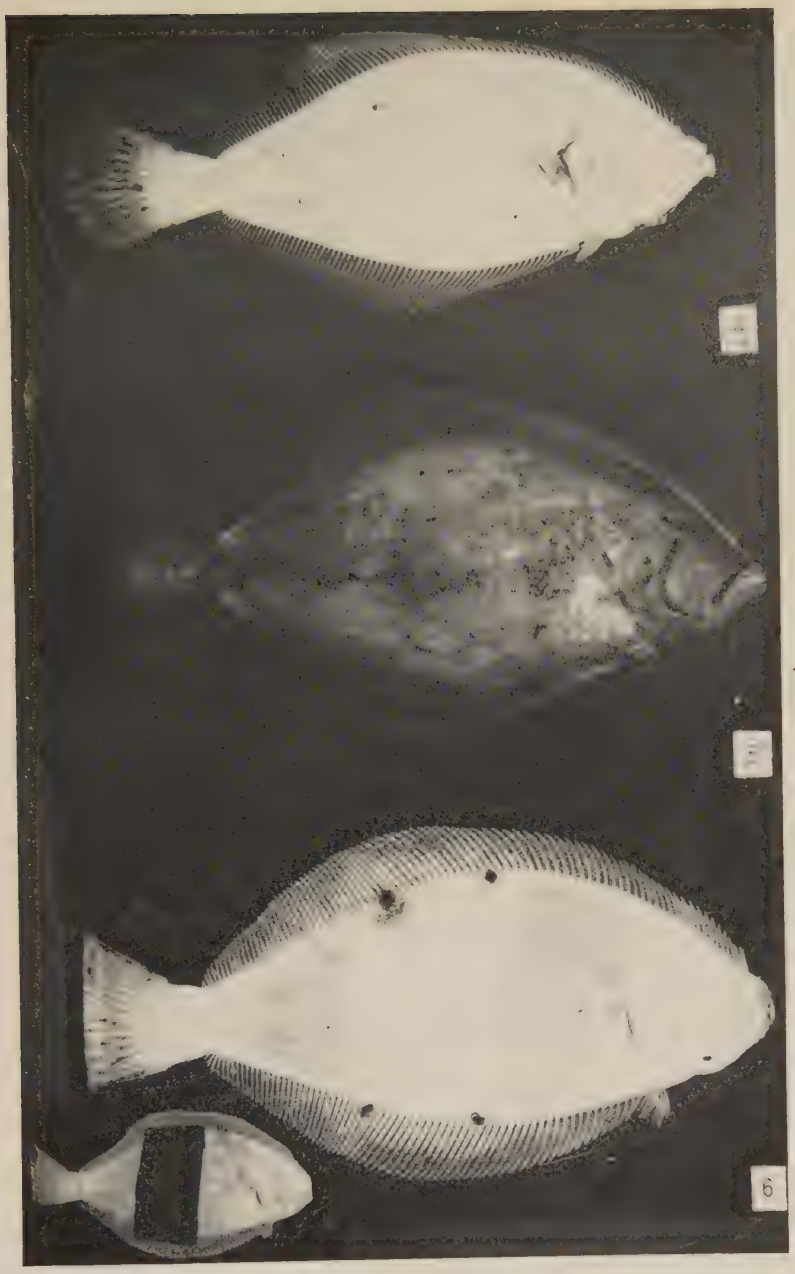

PLATE II.

All photographs are of the normally unpigmented ventral surface of living summer flounders. All inserts are further reductions of the corresponding animal.

FIG. 4. Summer flounder black-adapted 2½ days, blinded and placed in a brightly illuminated white tank (reflecting about 16 Weston units) for 28 days. A considerable increase in ventral melanophore production over that seen in the previous figures.

FIG. 5. Black-adapted 2 days, blinded and maintained in the special apparatus providing direct ventral illumination of high intensity (about 400 Weston units) for 26 days. First evidence of melanin formation was noted at about 90 hours. Note that the ventral surface has become quite completely darkened by an extensive covering of melanophores.

FIG. 6. This fish was maintained under the same conditions as described for Fig. 5. In addition an opaque mask was affixed to cover part of the ventral surface (see insert). This mask was kept in place during the entire sojourn of the animal in the ventral illumination apparatus (26 days). The mask was removed and the photograph taken at the end of that time. The area protected from the light is free from melanophores.

est. Fig. 3 and 4 illustrate typical cases.

Exp. 5. In this experiment intense light (400 Weston Units or 25X as much as was used in Exp. 4) was directed to the ventral surfaces of the fishes through glass bottomed tanks. This method provides two significant advantages. First, it is possible by using various lamps to subject the lower surface of the experimental animal to any intensity desired; and second, it is possible to measure with accuracy the amount of light falling directly upon the lower surface of the fish. Thus, illumination may be rigidly controlled.

Under such favorable conditions pigment formation proceeded rapidly. Frequently it was possible to detect some evidence of melanophore formation during the fourth day of illumination and after 4 weeks of such treatment the entire ventral surface was found to be generally darkened with an extensive growth of melanophores (Fig. 5).

Exp. 6. Success in blackening the ventral surface so completely with melanophores in a relatively short time suggested an experiment designed to determine whether the pigmentation response was due to the *direct* effect of illumination, assuming the internal environment to be optimum in all the experiments, or whether it was necessary only that light *be present* for the reaction to continue. It was reasoned, therefore, that if it were necessary for light to fall directly upon the cell (direct effect) areas covered by an opaque mask would remain unpigmented while exposed areas would develop melanophores as in Exp. 5. On the other hand if it was necessary only that a fish with optimum *internal* physiological conditions be generally illuminated one might expect that the pigmentation reaction would go on underneath the mask regardless.

Accordingly, flounders prepared as in Exp. 5 were further provided with opaque masks as described previously (Fig. 6—insert). They were illuminated ventrally (400 Weston Units) as before and simple, clean-cut results were obtained. The exposed surfaces became melaninated but the protected area remained as free from pigmentation as in the control fishes. This striking result seen in Fig. 6 seems to indicate clearly that light provides a direct stimulus to the particular cell destined to develop into a melanophore by producing melanin.

Discussion. It becomes clear from the data presented that light is not only a very significant factor in the experimental development of melanophores in a normally unpigmented area but also that there is a correlation between the amount of pigment grown, the intensity of the light and duration of the exposure. Data of a sufficiently quantitative nature to determine whether this relationship is direct over all ranges of light intensity are not available. It is probable, however, that a direct relationship between light intensity per unit time and degree of melanination would not hold in ranges of very high intensity. The physiological limit of the rate at which melanin could be formed would probably be reached before arriving at extremely high intensities of light.

The impression should not be gained from these experiments, however, that light is the most important single factor in the production of melanophores. Although it is obviously an essential factor (except perhaps in the case of regenerated scales) it must be emphasized that the condition inside the cell as evidenced by the degree of dispersion of melanin granules in the cells of normally pigmented areas must represent the darkening phase of the fish. The ineffectiveness of high intensities of light directed on the surface of a flounder while in a physiological condition not favoring darkening is strikingly shown in the case of a white-adapted fish where, instead of melanin production one witnesses an actual decrease in dark pigmentation due to melanophore degeneration.[12,13,22] The observation that in physiologically dark flounders regenerating scales develop melanophores even in the absence of illumination is further evidence that in certain instances light is not necessary for the growth of melanin in melanophores. The problem of the pigmentation of regenerating scales represents a special case which deserves more detailed investigation in the future.

Summary. 1. In addition to a favorable internal environment in the cell, light is necessary for the development of melanin in

melanophores of intact scales.

2. Light passing directly through glass bottomed tanks onto the lower surface of the flounders was the most effective in producing melanophores. This means of illumination delivered the most intense light to the ventral surface of the fish and could be most accurately measured.

3. The use of opaque masks indicates that light affects the cell directly to produce melanin.

4. The melanination of regenerating scales is apparently not dependent upon the presence of light. Investigation of this special problem is being continued.

16335

Technique for the Biomicroscopic Study of the Ovary and the Fallopian Tube.

CLEMENTE ESTABLE.

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In order to explore the ovary microscopically *in vivo* a method is described, the first step of which is the eventration of the ovary, with or without the Fallopian tube, and its placing in a subcutaneous loggia. To do this, the animal (rabbit) is fastened on its back, with the posterior part of its body rotated 30° to 45° on the longitudinal axis. Ether or some other anesthetic is administered. Then the ovary, with or without the Fallopian tube, is reached via a discreet section of the abdominal wall, slightly under the dorso-lateral line projected by the course of the Fallopian tube, and behind the kidney. Then the great external obliquus muscle is pierced by means of a surgical knife or the tip of a scissors slightly above this first incision, and the broad ligament is gently clamped; without being touched, the ovary is thus eventrated through the small opening in the muscle, the aponeurosis of which serves as a bed. All suturing close to the ovary should be avoided and is not necessary, as the ovary remains in its new position by simply placing it perpendicularly to the incision (Fig. 1). When removing the ovary from the abdomen great care must be taken not to provoke any circulatory or nervous disturbances; this point is of the utmost importance as regards all further activity of the ovary. The wound in the skin should not coincide with the ovarian surface or with the wound in the muscle which forms the bottom of the subcutaneous loggia. A flap-like portion of skin should cover the ovary, or the Fallopian tube, or both, as the case may be. Neither the suture of the muscle nor that of the skin should be in contact with the organs in the subcutaneous loggia. Thus, the ovary, the tube, or both can be easily reached without causing trauma. The best material for study is that in which no adhesion between skin and ovary arises; this is usually prevented by the secretions of the transferred organs, as well as by the frequent opening of the loggia necessary for repeated macro and microscopic studies of the ovary and the Fallopian tube.

Visualization capsule. The first experiment performed tried to keep the ovary visible through a grafted cornea. This procedure was a failure.

The visualization capsule is made up of 4 parts: (a) an ebonite ring of varying diameter and height, threaded on the inside and having a flat perforated base which allows it to be sutured under the skin, and large enough to accommodate the ovary and the Fallopian tube; (b) a second ring which screws into the first to which is fastened an extremely fine, transparent and elastic, removable diaphragm; 2 small holes in the ring

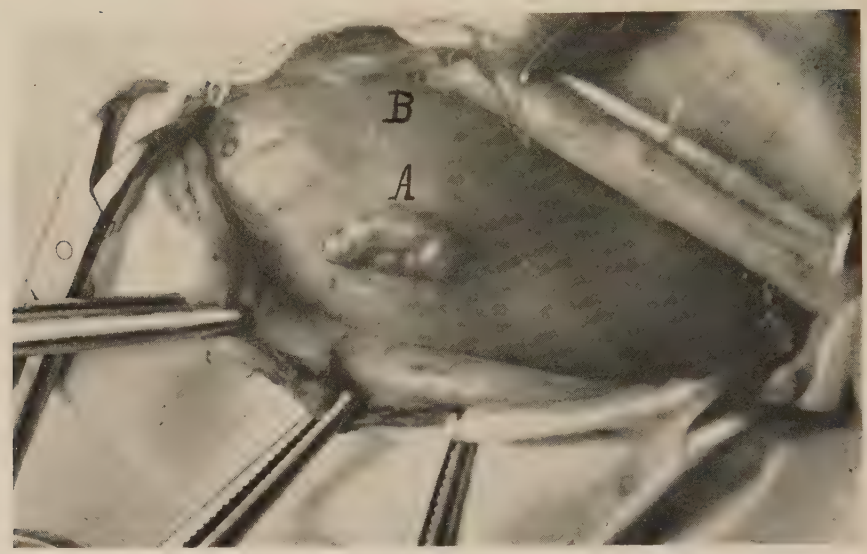

FIG. 1.
Ovary (A) transferred to the great obliquus muscle of the abdomen (B). The eventration was performed through a small opening. The ovary stays *in situ* without ligature or suture.

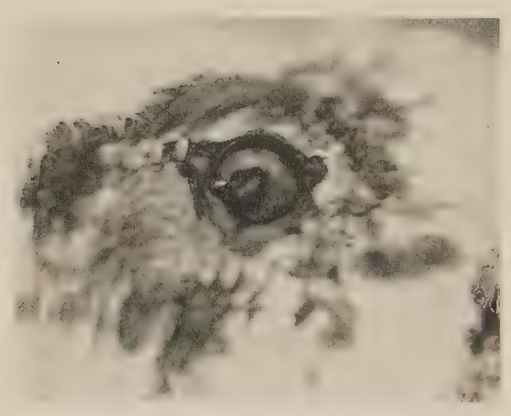

FIG. 2.
Eventrated ovary, within the fixed part of the visualization capsule.

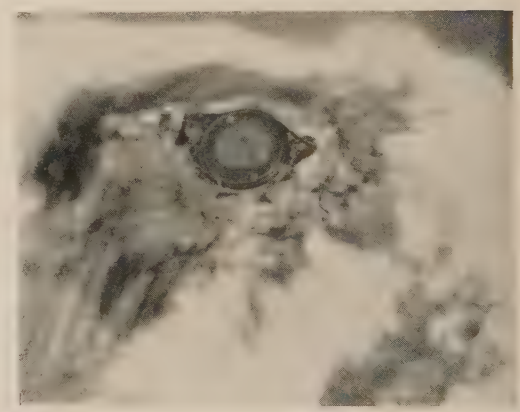

FIG. 3.
Ovary lying inside the visualization capsule, protected by a transparent, elastic diaphragm.

serve to screw or unscrew it; (c) an ebonite lid which screws onto the outside of the upper portion of the first ring, closing the ovarian chamber hermetically (Fig. 2, 3); it can be removed very easily every time it is necessary to make macro and microscopic observations of the ovary; (d) a metal key with 2 fine spigots which fit into the holes in parts (b) and (c) for screwing or unscrewing. This mobility and the elasticity of the diaphragm undoubtedly facilitate biomicroscopy and make preservation of the ovary very easy. The rigid diaphragm prevents the frontal lens of the objective from being brought as close to the ovarian surface as one would wish, becoming blurred more rapidly and being more difficult to replace. The construction allows the removal of parts and whenever necessary the examination of the ovary without any diaphragm. The lid protects the diaphragm and insures better asepsis; the capsule with the lid and without the dia-

phragm affords poor protection of the ovary against drying.

The subcutaneous ovary may be examined daily with the microscope,—the binocular, the ultrapaque or a mono-objective type; in the latter case a microlamp, an illuminating needle or a thin spatule of plastic material is placed directly beneath it or by perforating the tissues under the ovary.

When, through an error in operating, the hind leg of the rabbit rubs against the capsule, the sciatic nerve is sectioned to avoid irritation of the ovary, thus paralyzing the leg.

Precautions must be taken against vascular block,—as this might cause gangrene,—and against inflammatory irritations or damage to the nervous fibers. Obviously, this is not a subcutaneous graft of the ovary; it is a careful and adequate eventration, which maintains circulation and innervation intact.

With the technique for the biomicroscopic examination of the ovary and the Fallopian tube presented above, it is possible to follow, under the microscope, the development of the Graafian follicle, its circulatory changes, the exact moment when the "stigma" appears, its dehiscence, the flow of the follicular liquid carrying away with it the oöcyte, and the peristalsis of the Fallopian tube.

Observations already made are being prepared for publication.

Summary. For the microscopic observation *in vivo* of the mammal ovary and the Fallopian tube, two techniques are described, with special reference to the rabbit: (1) the transference of the ovary to a subcutaneous loggia, (2) the eventration of the ovary and its protection by a visualization capsule through which microscopic examination is possible. In both cases nervous and vascular connections are kept intact.

16336 P

Effect of Sodium Bicarbonate and Ammonium Chloride on Ascorbic Acid Metabolism of Adults.

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A consistent lowering of the urinary ascorbic acid excretion of adults has been observed when an increased urinary pH is effected by the ingestion of sodium bicarbonate.[1,2,3] Guinea pig experiments measuring "scurvy scores" and body stores of ascorbic acid[4] indicated that the lowered excretion of ascorbic acid as a result of the ingestion of sodium bicarbonate appeared to represent an increase in retention rather than greater destruction as might be supposed from *in vitro* experiments. This communication reports for the first time, as far as we know, the results of simultaneous daily determinations of plasma and urinary ascorbic acid concentrations of adults on controlled ascorbic acid intakes ingesting sodium bicarbonate or ammonium chloride.

Experimental. The subjects were 2 adult women, C. C. and J. G., in normal health, both actively interested in nutrition research. The experiment was divided into 3 periods of 14 days each: 1st period—basal diet[5] (containing by analysis 7 mg of ascorbic

* Present address, School of Home Economics, Oregon State College, Corvallis, Oregon.

1 Heinemann, M., *J. Clin. Invest.*, 1941, **19**, 39.

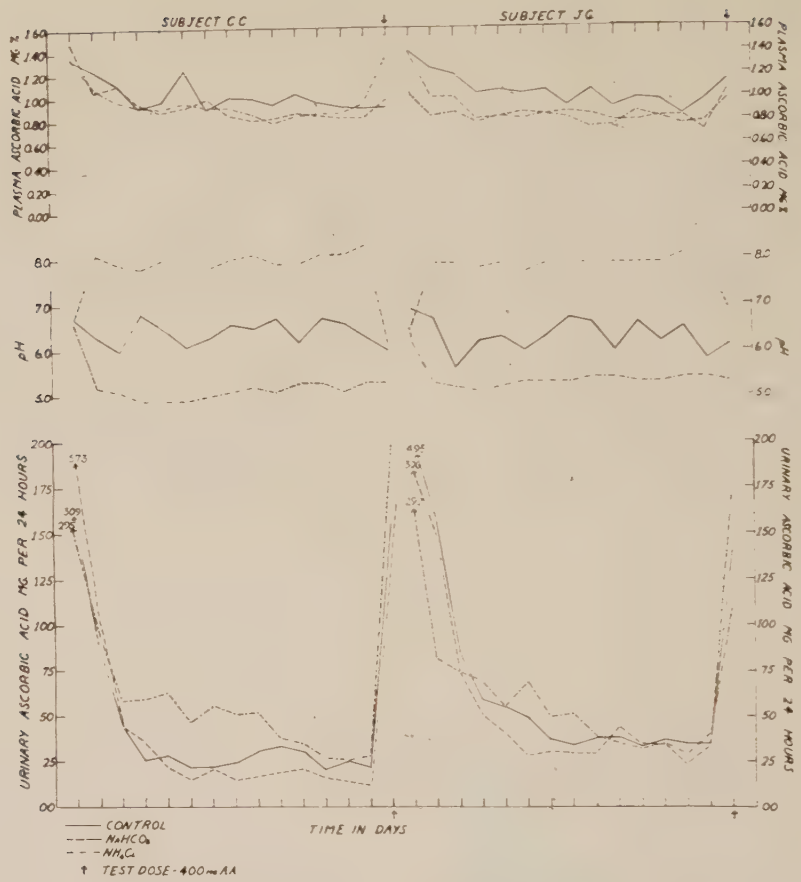
2 Hawley, E. E., Stephens, D. G., and Anderson, G., *J. Nutrition*, 1936, **11**, 135.

3 Patterson, I., unpublished data, Cornell University, Ithaca, N.Y., 1942.

4 Hawley, E. E., Daggs, R. G., and Stephens, D. J., *J. Nutrition*, 1937, **14**, 1.

5 Lewis, J. S., Storvick, C. A., and Hauck, H. M., *J. Nutrition*, 1943, **25**, 185.

FIGURE 1



THE EFFECT OF SUPPLEMENTS OF AMMONIUM CHLORIDE AND SODIUM BICARBONATE ON THE URINARY pH, URINARY EXCRETION OF ASCORBIC ACID AND PLASMA ASCORBIC ACID CONTENT OF TWO HUMAN SUBJECTS

FIG. 1.

acid) plus 60 mg of crystalline ascorbic acid daily; 2nd period—basal diet plus 60 mg of ascorbic acid plus 15 g $NaHCO_3$ daily; and 3rd period—basal diet plus 60 mg of ascorbic acid plus 4 g NH_4Cl daily. Preceding each experimental period the subjects were saturated with ascorbic acid[6] and each period was concluded with a determination of response to a 400 mg test dose. The 60 mg supplement of ascorbic acid was dissolved in water and taken just before breakfast; the 15 g of sodium bicarbonate were given as 5 g portions dissolved in water at 10 a.m., 3 p.m. and 8 p.m.; and the 4 g of ammonium chloride were given as 2 g portions dissolved in water at 10 a.m. and 3 p.m. The pH and ascorbic acid determinations were made on 24-hour urine collections according to the method described by Belser, Hauck and Storvick.[6†] Fasting plasma ascorbic acid values were determined by the method of Farmer and Abt.[7]

Results. The results are illustrated in Fig. 1. When compared with the control period, sodium bicarbonate decreased and

[6] Belser, W. B., Hauck, H. M., and Storvick, C. A., *J. Nutrition*, 1939, **17**, 513.

† The authors wish to acknowledge the assistance of Catherine Cobb and Josephine Graham for these determinations.

ammonium chloride increased the urinary excretion of ascorbic acid. The mean daily urinary ascorbic acid excretions for the 3 periods are: C. C. 1st period, 26±1.3 mg, 2nd period, 19±2.0 mg, and 3rd period, 44±4.1 mg; and J. G. 1st period, 41±2.7 mg, 2nd period, 34±7.6 mg, and 3rd period, 46±4.3 mg. The differences between the periods were significant for C. C. but not J. G. tested according to Livermore's formula: differences must be 2 times the standard deviation of the differences.[8] The mean daily plasma ascorbic acid levels were consistently and significantly[8] decreased from the levels of the regular control diet with the addition of sodium bicarbonate or ammonium chloride. The mean daily plasma ascorbic acid values for the 3 periods are: C. C. 1st period, 0.98±.03 mg %, 2nd period, 0.87±.02 mg %, 3rd period, 0.89±.02 mg %; and J. G. 1st period, 0.98±.02 mg %, 2nd period, 0.81±.04 mg %, and 3rd period, 0.79±.01 mg %.

Hathaway and Meyer[9] have defined "utilization" as the difference between intake and excretion. By this definition the subjects' apparent "utilization" on the 3 periods was as follows: C. C., 61%, 72%, and 34%, and J. G., 39%, 49%, and 31%, respectively. Apparent "utilization" is then increased with the administration of sodium bicarbonate and decreased with ammonium chloride ingestion. However, the lowered mean plasma ascorbic acid values and the varied plasma and urinary excretion responses to the administration of a test dose at the completion of each period (Fig. 1) are not consistent with the apparent percentage "utilization."

Summary. This study indicates that the changes in the excretion of ascorbic acid are not accurate indications of utilization. The ingestion of both salts significantly lowered the mean plasma ascorbic acid content of the subjects indicating an interference with normal utilization. The reduced excretion of ascorbic acid with the ingestion of sodium bicarbonate both daily and in response to a test dose would seem therefore to represent more accurately increased destruction of ascorbic acid in the excretory process; and conversely, the increased excretion with ammonium chloride would seem to represent increased preservation in the excretory process.

7 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 1625.

8 Hauck, H. M., personal communication, 1943.

9 Hathaway, M. L., and Meyer, F., *J. Nutrition*, 1941, **21**, 503.

16337

Application of a Metabolic Inhibitor to the Developing Chick Embryo.

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This research is the first of a series of attempts to apply a new method to the study of chemical embryology. The usefulness of metabolic analogs (anti-vitamins, anti-metabolites) in the study of the metabolism of microorganisms has been fully demonstrated by Shive and his co-workers in this laboratory by the development of the method of "inhibition analysis".[1,2] An outgrowth of these findings, which has been in prospect in this laboratory for some time, is an attempt to block metabolic reactions in embryonated and cancer-bearing eggs and to apply the same methods of study to these tissues. A study of the effect of precursors of the competing metab-

* Rosalie B. Hite Fellow.

1 Shive, W., and Macow, J., *J. Biol. Chem.*, 1946, **162**, 451.

2 Beerstecher, E., and Shive, W., *J. Biol. Chem.*, 1946, **164**, 53.

olite on such an inhibitor in embryonic tissue holds promise of information as to the embryo's biochemical abilities, as well as affording a means of following the development of the biochemical potentialities of the embryo in much the same manner that the morphological development has been traced.

The inhibitor, 3-acetylpyridine, used in this investigation has already found use in the production of pellagra-like symptoms in the mouse, an animal normally requiring no exogenous source of nicotinic acid.[3] It has been reported that its toxicity on this animal can be nullified by feeding nicotinic acid or tryptophan which have been postulated as precursors of nicotinamide.[4,5]

Developing chick embryos are inhibited by 3-acetylpyridine and this inhibition can be reversed competitively by nicotinamide, but nicotinic acid and tryptophan are much less active reversing agents. Details of some of these experiments with embryonic chicks and their implications are presented below.

Testing Methods. All solutions injected into eggs were adjusted to a pH of 7 and were equivalent in osmotic pressure to .58% saline. Sterilization was accomplished by autoclaving and the same precautions of aseptic technique were used in yolk-sac injection as are described for tumor in egg cultivation.[6] Deaths were determined by candling. Time and temperature of incubation are given in the tables.

Results. The results shown in Table I indicate that 600 γ of 3-acetylpyridine per egg is lethal in 24 hours when injected into the yolk-sac. Sub-lethal doses of 3-acetylpyridine or lethal doses rendered just sub-lethal by the addition of nicotinamide or precursors of this vitamin, cause a mal-development of the chick. Some of the symptoms associated with the toxicity were undersized deformed legs, and a general edematous-like condition over the surface of the body. These symptoms could be prevented by the simultaneous injection of large amounts of nicotinamide with the 3-acetylpyridine.

Reversal of the Lethal Effect. The amount of nicotinamide necessary to just nullify the lethal effect of 3-acetylpyridine increases as the concentration of this inhibitor increases. Further, from Table I it can be seen that the relationship of these 2 substances is a competitive one and that the inhibitor-substrate (3-acetylpyridine-nicotinamide) ratio is approximately 14. In order to determine this ratio, it is necessary to measure the total inhibitor and substrate which is just non-lethal to the egg. However the egg itself contains some nicotinamide and for this a correction must be made by deducting from the total 3-acetylpyridine that amount which corresponds to the maximum non-lethal dose.

The justification for determining the inhibition constant in this manner can be demonstrated concisely if we allow:

I = Total inhibitor
S = Total substrate
S_a = Added substrate
S_o = Substrate present in egg initially
I_o = Maximum non-lethal level of inhibitor
E = Enzyme
K = Inhibitor-substrate ratio
P = Product of substrate

The competition of inhibitor and substrate for some specific enzyme has been represented by the following expressions:

$$S + E \rightleftharpoons ES \rightarrow E + P$$
$$I + E \rightleftharpoons EI$$

For such a competitive system by definition, the ratio of I to S is a constant

$$(1) \qquad \frac{I}{S} = K$$

Since S represents the total amount of nicotinamide present, and this is composed of the vitamin already in the yolk plus that which has been injected then:

$$(2) \qquad S = S_a + S_o$$

From expression (1) and (2) one obtains:

$$\frac{I}{S_a + S_o} = K$$

or by rearrangement:

$$(3) \qquad I = KS_a + KS_o$$

Now from equation (3) it is obvious, that when the added substrate is zero, the amount of inhibitor which the eggs will tolerate is

[3] Woolley, D. W., *J. Biol. Chem.*, 1945, **157**, 455.
[4] Woolley, D. W., *J. Biol. Chem.*, 1946, **162**, 179.
[5] Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1946, **163**, 343.
[6] Taylor, A., Thacker, J., and Pennington, D., *Science*, 1942, **96**, 342.

TABLE I.
Effect of 3-Acetylpyridine and Nicotinamide on 4-day-old Chicks.

Nicotinamide γ per egg	3-Acetylpyridine γ per egg	No. of eggs* injected	Exper. fraction dead in 24 hr	Control† fraction dead in 24 hr	$\frac{I - I_o}{S_a}$‡
—	400	8	0.00	0.00	—
—	450	8	0.13	0.00	—
—	500	7	0.14	0.00	—
—	550	7	0.14	0.14	—
—	600	8	1.00	0.00	—
80	2000	7	0.72	0.00	—
90	2000	7	0.43	0.00	—
100	2000	7	0.00	0.00	14.5
160	3000	7	0.72	0.00	—
180	3000	7	0.00	0.00	13.6
200	3000	7	0.00	0.00	—
200	4000	8	1.00	0.00	—
220	4000	8	0.63	0.00	—
230	4000	7	0.57	0.14	—
240	4000	8	0.00	0.00	14.4
280	5000	8	0.50	0.00	—
313	5000	8	0.00	0.00	14.2
325	5000	8	0.00	0.00	—
350	6000	8	1.00	0.00	—
370	6000	8	0.75	0.00	—
380	6000	8	0.00	0.00	14.3

* 4-day-old eggs were used, incubated at 37°C.
† Control eggs were injected with .58% sterile saline.
‡ These values were calculated; I and S correspond to the inhibitor and substrate added and I_o is the maximum non-lethal dose experimentally determined as 550 γ per egg.

K S_o and represents the largest non-lethal dose. Designating S_oK as I_o and substituting in expression (3) one has by rearrangement:

$$\frac{I - I_o}{S_a} = K \qquad (4)$$

In this last equation the inhibitor-substrate ratio, K, is expressed in all experimentally measurable quantities.

From the experimental value of I_o and the average experimental value of K, the quantity of S_o can be estimated, since by designation in expression[4]

$$I_o = S_oK$$

The value of S_o so estimated for eggs averaging 56 g was found to be approximately 39 γ. The value of Cheldelin and Williams[7] determined by microbiological assays is .72 γ per gram or 40.3 γ per 56 g eggs.

7 Cheldelin, V. H., and Williams, R. J., *The University of Texas Publication*, 1942, No. 4237, 105.

Effect of Nicotinic Acid and Tryptophan. The effects of nicotinic acid and tryptophan on the toxicity of 3-acetylpyridine on 4-day-old embryos is shown in Table II. It is striking that these two substances while active against low levels of inhibitor are much less potent than the nicotinamide. This does however demonstrate that the 4-day-old chick has the mechanism for the biochemical conversion of nicotinic acid and tryptophan to nicotinamide and that the ability is very feeble. This might indicate that at this stage of development the concentration of the enzymes necessary for the conversion of nicotinic acid to its amide is very low.

Summary. 1. Embryonic chicks were inhibited by the direct injection of 3-acetylpyridine into the yolk-sac. 2. It was shown that this inhibition could be reversed competitively by nicotinamide over the range of 2000 to 7000 γ per egg. 3. Evidence is presented showing that 4-day-old chick embryos have

TABLE II.
Effect of Nicotinic Acid and Tryptophan on the Toxicity of 3-Acetylpyridine.

3-Acetylpyridine γ per egg	Nicotinic acid γ per egg	Tryptophan γ per egg	No. of eggs* injected	Fraction dead in 24 hr
1500	0	0	6	1.00
1500	2000	0	6	0.50
1500	2500	0	6	0.67
2000	0	0	7	1.00
2000	2000	0	7	1.00
2000	2500	0	7	0.88
600	0	0	7	0.86
600	0	7500	7	0.00
650	0	0	7	1.00
650	0	7500	7	0.00
700	0	0	7	1.00
700	0	7500	7	0.14

* All eggs incubated at 37°C and were 4 days old at time of injection.

a very limited capacity to convert nicotinic acid to the corresponding amide. 4. Sub-lethal levels of 3-acetylpyridine are shown to cause a mal-development of the chick embryo.

16338

Effect of pH on MM Virus.

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MM virus was originally isolated from a hamster which had been injected intracerebrally with cord and medulla of a fatal case of poliomyelitis. Typical and severe poliomyelitic lesions are induced by it in the central nervous system of hamsters and albino mice.[1]

Serologically MM virus appears to be similar to the SK virus of Jungeblut and Sanders[2] but differs from the Lansing-like poliomyelitic strains and the Theiler virus.[3]

The virus is highly virulent for mice by the intraperitoneal as well as the intracerebral route. Thus it affords an opportunity for testing the effects of antibiotic agents on small amounts of a virus which induces poliomyelitic lesions in the test animal. In relation to a problem of this kind[4] an investigation was made of the effect of pH on MM virus.

Procedure. Mouse brains from the 45th to the 48th generation stored in 50% glycerol were ground, made up in a 10% suspension usually in salt solution containing 10% of infusion broth or occasionally in distilled water, and centrifuged to remove large particles. The supernatant fluid was used at once or after

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1 Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

2 Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

3 Dalldorf, G., to be published.

4 Schatz, A., and Plager, H., *Bull. Torrey Bot. Club*, 1948, **75**, 256.

storage in the dry-ice box. Albany standard strain mice† weighing 10 to 12 g were injected intraperitoneally. By this route of inoculation the majority of animals given 0.05 ml of a 0.5 x 10^{-6} dilution of mouse brain were usually dead or paralyzed by the seventh day. Unless otherwise specified, animals were observed over a period of one week after which experiments were discontinued. Five mice were employed for each individual test.

The buffers were Michaelis' veronal acetate,[5] Sorenson's glycocoll sodium hydroxide,[6] and the phthalate, phosphate, borate, and hydrochloric acid mixtures of Clark and Lubs.[6] These were made up approximately to the desired pH and sterilized by heating for 10 to 15 minutes in a water bath or by autoclaving for 15 minutes at 15 pounds' pressure. In most instances, 0.5 ml of a 10^{-3} distilled-water dilution of virus-infected mouse brain was added to 4.5 ml of buffer. The effect of distilled water and of physiologic saline adjusted to different reactions with acetic or hydrochloric acid was also determined. After the desired periods of incubation at room temperature, dilutions were made in broth-salt solution and tested in mice.

Final pH values for the suspensions were determined electrometrically on corresponding normal mouse-brain mixtures, except for the first test with veronal acetate and glycocoll solutions in which the effect of buffer dilution was duplicated with distilled water instead of normal mouse-brain suspension. Bromphenol and bromthymol blue indicator tests with 10^{-4} dilution of virus mouse brain and normal mouse brain in veronal acetate buffer at pH 4.0 and 7.0 and also in aqueous suspensions at pH 7.4 revealed no difference in reaction between the normal and infected mouse-brain suspensions. For solutions at pH 9.0 and above, corrections for sodium-ion

† A strain used at the Division of Laboratories and Research, New York State Department of Health, Albany, New York.

[5] Michaelis, L., *Biochem. Z.*, 1931, **234**, 139.

[6] Clark, W. M., *The Determination of Hydrogen Ions*, Baltimore, Williams and Wilkins, 1928, 717p.

TABLE I. Survival of MM Virus in Veronal-acetate and Glycocoll NaOH Buffers. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	Exp. No.	pH	Period of exposure, days: 1 — Virus dilutions inoculated into mice: 10^{-4}	1: 10^{-5}	1: 10^{-6}	2: 10^{-5}	2: 10^{-6}	2: 10^{-4}	7: 10^{-5}	7: 10^{-6}
Veronal-acetate buffer	2	2.02	0/5	0/5	0/5					
" "		3.48								
" "	2	4.05	2/5*	0/5	0/5			0/5	0/5	
" "	1	4.15		0/5	1/5	2/5	0/5	0/5	0/5	
" "	1	5.17		0/5	0/5	0/5	0/5	0/5	0/5	
" "		6.18								
" "	1	7.15		5/5	5/5	5/5	5/5	5/5	5/5	4/5
" "	1	8.04		5/5	5/5	5/5	5/5	5/5	5/5	5/5
" "	1	8.95		5/5	4/5	5/5	5/5	5/5	5/5	5/5
Glycocoll-NaOH buffer	1	8.99		5/5	5/5	4/5	3/5	5/5	5/5	5/5
" "	1	9.95		5/5	5/5	5/5	4/5	3/5	1/5	0/5
Control (distilled water)	1			5/5†	5/5‡	5/5	5/5		5/5	5/5
" "	2	7.40		5/5	3/5				5/5	3/5

Numerator = number of mice which died; denominator = number of mice injected. Test period = 7 days.
* 2 survivors paralyzed on 7th day. † 2 paralyzed mice sacrificed on the 3rd day. ‡ 1 paralyzed mouse sacrificed on the 3rd day.

TABLE II. Survival of MM Virus in HCl, Phthalate, Phosphate, and Borate Buffers. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	Exp. No.	pH	Period of exposure, days: 1 — Virus dilutions inoculated into mice: 10^{-4}	10^{-5}	10^{-6}	2 — Virus dilutions inoculated into mice: 10^{-4}	7 — Virus dilutions inoculated into mice: 10^{-4}	10^{-5}	10^{-6}
HCl	3	1.12	0/5	0/5	0/5				
	3	2.10	1/5	0/5	0/5				
Phthalate buffer	3	3.00	0/5	0/5	0/5				
	3	4.00	1/5	0/5	0/5				
	3	5.00	0/5	0/5	1/5				
Phosphate buffer	4	6.00		3/5	1/5	2/5	0/5	0/5	
	4	7.01		5/5	5/5	5/5	5/5	5/5	5/5
	4	7.75		5/5	5/5	4/5	3/5	4/5	0/5
Borate buffer	4	7.71		4/5*	5/5	4/5	4/5	5/5	4/5
	4	8.75		5/5	4/5	5/5	5/5	5/5	5/5
	4	9.55		5/5	4/5	3/5	3/5	0/5	0/5
Control (distilled water)	3	7.01		4/5	3/5			5/5	2/5
	4	7.47		5/5	5/5	5/5	3/5*	5/5	3/5

Numerator = number of mice which died; denominator = number of mice inoculated. Test period = 7 days.
* 1 survivor paralyzed. † ± 0.05.

concentration were taken into account. Inoculation of mice with virus-free buffers at the extremes of pH indicated no toxicity for the solutions *per se*. When streaked on blood-agar plates, the mixtures gave no growth.

Experimental Results. Tables I and II indicate the pH tolerance of MM virus in the different buffers at room temperature. These results and other data not presented here show that under the conditions of the experiment the virus when exposed at 10^{-4} dilution was stable in the neutral and alkaline solutions. There was a definite loss of activity within twenty-four hours in the veronal-acetate and phosphate buffers below pH 7. On the other hand, the virus survived for at least 2 days in glycocoll and borate solutions at pH 9.95 and 9.55, respectively, but a decrease in activity was evident when tested on the 7th day. The veronal-acetate buffer, at approximately pH 7.8, contained infective virus in the 10^{-6} dilution after seven days at room temperature followed by refrigeration for 8 days. There was no significant difference between the phosphate and phthalate buffers at pH 6.0. However, the results did indicate that virus survival was somewhat better at around pH 8.0 in the borate than in the phosphate medium; at 10^{-4} dilution, active virus was present in the former but not in the latter buffer after 7 days at room temperature followed by 10 days at 4°-6°C.

In a more detailed experiment, ten-fold virus dilutions from 10^{-2} through 10^{-6} or 10^{-7} in broth salt solution, were exposed to equal amounts of veronal-acetate buffers. Similar dilutions prepared with normal mouse brain had pH values of 4.35, 5.25, 6.35-6.45, 6.85-6.95, 7.75-7.8. After incubation for 1- and 7-day periods, suitable ten-fold dilutions were inoculated into mice. The results indicated, in general, a less deleterious effect of unfavorable pH in the more concentrated virus suspensions. The virus stability was definitely greater at pH 7.8 than at pH 6.9. In this experiment and in several others, there was occasional evidence of some virus survival at around pH 4.0 as compared to that at pH 5.0. While these results are suggestive of greater virus stability somewhere

in the vicinity of pH 4.0 than at acid reactions immediately above or below, more detailed studies would be required to ascertain whether this is actually so.

Whether the increased virus survival in the lower dilutions and the apparent occasional survival at approximately pH 4.0 were due to a protective action of the mouse-brain constituents is not known. No visible flocculation of the mouse-brain suspensions occurred in the buffers with 10^{-4} dilution. However, at a dilution of 10^{-2}, maximum precipitation was observed at pH 3 to 5. This is in agreement with a similar observation of Wenner[7] on flocculation in mouse-brain virus suspensions at pH 4.0 to 5.5.

The effect of acidifying MM virus suspensions immediately before inoculation was also studied. Hammon and Izumi[8] using the Armstrong mouse-adapted poliomyelitis virus and Wenner with the Lansing strain reported that the mortality rate for mice inoculated with mouse suspensions at pH 4.0 and 4.6 respectively, was higher than the death rate of animals given neutral or alkaline inocula. In order to determine whether the MM strain was affected by readjustment before inoculation, 10^{-3} and 10^{-5} dilutions of the virus were exposed to veronal-acetate buffers of pH 4, 6, and 8; the broth-salt control was at pH 7.2. After incubation for 1- and 24-hour intervals, a portion of each virus-buffer suspension was adjusted with 0.4% NaOH to about pH 7.5. These alkalinized suspensions, as well as samples of the corresponding unadjusted virus-buffer mixtures, were inoculated into mice within an hour. The results indicated that readjustment to the favorable reaction range had no beneficial or potentiating action. In these experiments light was not excluded during the exposure periods.

In other tests, however, in which distilled water, physiologic saline, and buffer solutions were compared, precautions were taken to protect the test material from possible effects of light. Under these conditions, results with

TABLE III.

Survival of MM Virus in Acidified Distilled Water and Saline and in Veronal-acetate Buffer. 10^{-4} Virus Dilution Exposed at Room Temperature.

Diluent	pH		Period of exposure, days							
			1				7			
			Virus dilutions inoculated into mice							
	1 day	7 days	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Distilled water + HCl	3.6	3.7	4/5	4/5	3/5*	1/5	5/5	0/5	2/5	0/5
" " "	5.65	5.85	5/5	5/5	1/5	0/5	4/5	0/5	0/5	0/5
Distilled water	6.1	6.1	5/5	5/5	5/5	2/5	5/5	2/5	0/5	0/5
Saline + HCl	3.75	3.9	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Saline	6.1	6.25	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Veronal-acetate buffer	4.0	4.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
" " "	6.0	6.0	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
" " "	7.25	7.2	5/5	5/5	4/5	1/5	5/5	3/5*	0/5	0/5

Numerator = number of mice which died; denominator = number of mice inoculated. Test period = 7 days.
* 1 survivor paralyzed.

[7] Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 104.

[8] Hammon, W. M., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 579.

veronal-acetate buffers were similar to those previously obtained. In the range tested, from about pH 4.0 to pH 6.0, physiologic saline had an effect similar to that noted with veronal-acetate buffer solutions. On the other hand, the survival of virus in distilled water over the same range was apparently greater. Material diluted to 10^{-6} after exposure for 24 hours in a 10^{-4} dilution at about pH 4.0 was lethal for mice. After exposure for one week virus had survived in the 10^{-4} dilution close to pH 4.0 (Table III).

Summary. In various buffers, MM virus was stable at room temperature only at neutral or alkaline reactions. When diluted virus was exposed at approximately pH 1.0 to neutrality the infectivity was diminished within twenty-four hours. There was a slower decrease in virus titer at pH 9 to 10. At pH 7 to 9, the virus was found to be stable for more than seven days. The deleterious effect of unfavorable pH was less for concentrated virus suspensions. MM virus was apparently more stable in distilled water acidified with hydrochloric or acetic acid than in acidified physiologic saline or in veronal-acetate buffer solutions of approximately the same pH value.

16339

Experimental Enterococcal Food Poisoning in Man.

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Several outbreaks of food poisoning of a mild type in which enterococci were the predominant organisms isolated from the suspected foods, are described in a previous paper.[1] The literature on food poisoning caused by non-hemolytic streptococci is also discussed and it is pointed out that, in several instances in which the inculpated organisms were closely identified, they were found to be enterococci.

The relatively small number of outbreaks reported as caused by *Streptococcus faecalis* is in sharp contrast to the widespread occurrence of this organism in nature.[1] Accordingly the studies reported in this paper were initiated in an attempt to determine whether *Streptococcus faecalis* can act as a food poisoning agent. They consisted of feeding experiments with *Streptococcus faecalis* employing kittens and human volunteers.

The literature contains the following references to experimental streptococcal food poisoning in man. Cary, Dack, and Meyers[2] reported that a volunteer who had eaten parts of several sausages similar to others which had been implicated in an outbreak of mild gastroenteritis, developed nausea which increased for 24 hours, with two periods of vomiting and epigastric pain, followed by marked exhaustion and constipation. A green "pleomorphic" streptococcus whose species designation is not determinable from the description given was isolated from the sausage. Later Cary, Dack, and Davison[3] reported abdominal distress, cramps, and diarrhea, in 5 of 7 volunteers who ingested "reasonable" doses of several strains of alpha streptococci which had been implicated in 2 outbreaks of food poisoning. On the other hand, Dolman[4] reported failure to produce food poisoning in any of 4 volunteers each of whom had eaten a meat pie heavily infected with *Streptococcus "viridans"* which had been isolated from

[1] Buchbinder, L., Osler, A. G., and Steffen, G. I., *Pub. Health Rep.*, 1948, **63**, 109.

[2] Cary, W. E., Dack, G. M., and Meyers, E., PROC. SOC. EXP. BIOL. AND MED., 1931, **29**, 214.

[3] Cary, W. E., Dack, G. M., and Davison, E., *J. Inf. Dis.*, 1938, **62**, 88.

[4] Dolman, C. E., *Canad. J. P. H.*, 1943, **34**, 97.

rissoles implicated in a food poisoning outbreak.

Some success in producing experimental *Streptococcus faecalis* food poisoning in man is reported in the present paper.

Experimental. Studies with Kittens. Studies were carried out with 70 kittens in which evaporated milk containing 20-hour cultures (test animals) or evaporated milk alone (control animals) was fed. This line of investigation was found to be unsatisfactory and abandoned because it was noted that the controls occasionally developed diarrhea.

Studies with Human Subjects. All persons who served as subjects for these studies had undergone physical examination and gave no history of recent gastro-intestinal disturbance. Volunteers were given food which had been experimentally contaminated with *Streptococcus faecalis.*

Feeding with 20-hour Cultures. Attempts to produce experimental gastroenteritis with 20-hour cultures of *Streptococcus faecalis* were not fruitful. Twenty-one volunteers were fed 2.5 to 50 ml of 20-hour cultures of this streptococcus grown in milk or infusion broth. In no instance was a definite food poisoning syndrome provoked although 4 subjects complained of slight nausea which persisted in one individual for 24 hours. There were no such complaints among 11 volunteers who drank the vehicle without organisms. The reason for the failure of these 20-hour cultures to produce symptoms is not apparent.

Feeding with 5-hour Cultures. Four strains of *Streptococcus faecalis* were used. Three of these (A4, B3, JA2) had been isolated from human feces within 2 months of their use; the fourth, A34.1, had been isolated about one year previously from a can of evaporated milk implicated in an outbreak of gastroenteritis.

Three different foods were used; they were egg salad, custard, and sterile milk. Each food was prepared in average sized individual portions. The portions were inoculated with 5.0 ml of a 20-hour infusion broth culture, incubated at 37°C for 5 hours and then served. The egg salad portion consisted of the amount needed for one sandwich, the custard portion of one cup, and the milk of 50 ml which was diluted with sterile milk to make up one glassful. Foods used for control purposes were prepared and incubated in the same manner as were the test foods except that no cultures were added to them.

All feedings were given shortly before noon. There was no marked difference in taste between the treated and untreated foods and the volunteers were unaware of the status of the food which each consumed. The volunteers limited their subsequent luncheon to lettuce and tomato sandwiches, tea or black coffee, and fruit.

When symptoms occurred after the experimental feeding the diagnosis was made by a physician who did not know the test status of the individual concerned.

Results and Discussion. The findings are recorded in Table I. It is noted that 6 of the subjects who ingested food contaminated with cultures of *Streptococcus faecalis* had definite symptoms of food poisoning, while a seventh had questionable ones. Nineteen volunteers who ate the same contaminated foods developed no symptoms, while 18 others who received the same foods but without addition of the specific bacteria, likewise, remained negative. Only 2 of the 4 strains used provoked symptoms of food poisoning. One of these was a recently isolated fecal strain and the other had been recovered about one year previously from food implicated in an outbreak. Thus 6, possibly 7, of 17 persons who ingested these strains became ill. Some of those who showed no symptoms ingested larger doses than others who did become ill. The production of symptoms in only a fraction of the subjects is paralleled by somewhat similar findings in some naturally occurring outbreaks of food poisoning apparently induced by *Streptococcus faecalis.*[1] The 2 strains which failed to produce symptoms were tested on a total of 9 persons.

The use of 5-hour cultures in this experiment was based on observations of the growth rates of these organisms in broth culture which indicated that good growth had occurred in that time and also, perhaps more

TABLE I.
Food Poisoning in Human Volunteers.
Ingestion of Milk, Custard, and Egg Salad Inoculated with *Streptococcus faecalis* and Incubated at 37°C for 5 hr.

	Cultures	Quantity of food	No. of organisms per g ($\times 10^6$)	Test			Control (food without culture)	
				No. of persons fed	No. of persons ill	Case	No. of persons fed	No. of persons ill
Milk	A4, A34.1, B3	50 ml	454-990	3	1	A	2	0
Custard	A4, A34.1, B3, JA2	92-94 g	168-1,050	12	2	B, C	8	0
Egg Salad	A4, A34.1, B3, JA2	41-87 g	740-2,100	11	4 (1?)	D, E, F, G(?)	11	0

	Case	Culture	Dose	Symptoms
Milk	A	A4	22.7×10^9	Nausea and regurgitation which began after 2½ hours and lasted for 24 hours. Slight diarrhea after 9 hours.
Custard	B	A34.1	45×10^9	Nausea, diarrhea and repeated vomiting which began 2 hours after feeding and persisted for 32 hours.
	C	A34.1	79.2×10^9	Nausea, eruction and mild diarrhea. Symptoms began after 2 hours and persisted for 37 hours.
Egg Salad	D	A4	35.7×10^9	Nausea and vomiting, dizziness and abdominal cramps which began after 2 hours and lasted for 30 hours.
	E	A34.1	107×10^9	Nausea, abdominal cramps and one bout of vomiting which began after 10 hours and lasted for 2 hours.
	F	A4	148×10^9	Diarrhea after 8 hours.
	G (?)	A34.1	99.6×10^9	Nausea which began after 2 hours and lasted for 12 hours.

significantly, on the knowledge that incubation periods of such duration of suspected foods are frequently found in food poisoning outbreaks. It was found that 10 to 25 fold increases of initially large inoculums had taken place in the vehicle foods prior to ingestion.

It is of interest that the number of organisms which were found to produce experimental symptoms in this study is of the same order as that which Kelly and Dack[5] found able to produce symptoms of gastroenteritis when a strain of *Staphylococcus aureus* was fed. These authors produced illness in one volunteer with 48×10^9 organisms and in another with 69.3×10^9. In the present study the symptom producing dose of *Streptococcus faecalis* ranged from 22.7×10^9 to 148×10^9 organisms. This comparison is noteworthy since staphylococcal food poisoning is believed to be caused by an "enterotoxin" while Sherman, Gunsalus, and Bellamy[6] suggest the possibility that the toxic principle of *Streptococcus faecalis* poisoning may be tyramine formed by the decarboxylation of tyrosine by this organism. Also perhaps worthy of comment is the fact that like staphylococcal food poisoning, streptococcal food poisoning usually has a short incubation period.

Summary. Symptoms of acute gastric or intestinal disturbance or of both were produced in 6, or possibly 7, of 26 human volunteers who ate foods in which strains of *Streptococcus faecalis* had grown for 5 hours.

Attempts to produce similar symptoms in man with 20-hour cultures were unsuccessful. Kittens were found to be unsatisfactory test animals in feeding experiments with inoculated condensed milk.

Conclusion. The experimental production of acute mild intestinal or gastric disturbance or both in man, tends to confirm the etiological role of *Streptococcus faecalis* in naturally occurring outbreaks of food poisoning.

We are indebted to Drs. William Birnkrant and Morris Greenberg of the Bureau of Preventable Diseases who conducted the physical examinations and diagnosed the illnesses which occurred.

5 Kelly, F. C., and Dack, G. M., *A. J. P. H.*, 1936, **26**, 1077.

6 Sherman, J. M., Gunsalus, I. C., and Bellamy, W. D., 57th Annual Report, N. Y. State Col. Agr., Cornell University Agr. Exp. Sta., 1944.

16340 P

Distribution and Nature of the "Antigen" Responsible for Experimental Meningoencephalomyelitis in the Guinea Pig.*

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Previous work has shown that a meningoencephalomyelitis, sometimes with foci of demyelinization, may occur in monkeys, guinea pigs, and rabbits 2 to 9 weeks following the subcutaneous or intramuscular injection of brain tissue mixed with acid-fast bacilli in a water-in-oil emulsion.[1-6] In con-

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† The able assistance of Mr. Robert H. Purnell, Cornell University Medical College, is gratefully acknowledged.

1 Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131 (preliminary report in *J. Bact.*, 1946, **51**, 53).

2 Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117 (preliminary report in *Science*, 1946, **104**, 362).

3 Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neur.*, 1947, **6**, 333.

4 Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

5 Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229.

firmatory work in this laboratory an ascending paralysis has developed in slightly more than half of some 90 guinea pigs following the single subcutaneous injection of one cc of similar emulsions containing guinea pig, rabbit, human, or beef brain suspended 1:20 in 0.9% saline. The illness usually began 15 to 31 days after injection, although it was delayed as long as 74 days in one case; the animals usually died within 10 days, although some recovered and others remained paralyzed for one to 2 months. Histological examinations of brain and spinal cord removed from about 30 paralyzed guinea pigs in every case revealed a patchy or diffuse perivascular inflammation, predominantly lymphocytic, in the leptomeninges and entire central nervous system, most concentrated in the white matter. The evidence that this disease may represent an allergic reaction has been recently reviewed.[7] Further experiments now to be described have shown that the "antigen" responsible for this experimental disease is present in certain phosphatide-like extracts of brain tissue, in a preparation of "purified brain lipids,"‡ and in optic nerve, which contains only white matter.

To learn about the nature of the "antigen," various extracts of whole moist beef or human brain were made according to the outline of Page,[8] using acetone, cold petrol ether, cold 95% alcohol, ether, and warm pyridine. In all, 5 different preparations of phosphatide-fractions were made, these being insoluble in acetone and soluble in cold petrol ether and ether; 4 of these were further fractionated into a lecithin-component (soluble in cold 95% alcohol) and a cephalin-portion (insoluble in cold 95% alcohol). Eight different preparations of other fractions of brain were made. These included cholesterol and other substances repeatedly soluble in acetone; cerebrosides and sphingomyelin or a mixture of these referred to as "protagon,"[9] which were insoluble in acetone, cold petrol ether, and cold 95% alcohol but soluble in pyridine warmed to 30°-45°C and in alcohol warmed to 80°C; and residual material which was insoluble in acetone, cold petrol ether, and warm pyridine. Each of these fractions was made up to an approximately 5% suspension in 0.9% saline; an emulsion was then made by mixing two parts of this suspension in a Waring blendor with two parts of Bayol-F§ containing 2.5 mg/cc heat-killed tubercle bacilli and one part of Falba.‖ Each emulsion was then tested by injecting one cc subcutaneously into each of 3 guinea pigs. Four of the 5 emulsions containing the phosphatide-fractions produced paralysis in 6 of 13 guinea pigs injected, whereas none of the 8 emulsions containing the other fractions produced any illness in 22 guinea pigs injected.

As a check on the lipid nature of the "antigen," an emulsion of "purified brain lipids"‡ was made and injected into 5 guinea pigs. Nine days following a second injection 3 months later, one guinea pig developed a characteristic paraplegia. None of 5 guinea pigs similarly injected twice with an emulsion containing "purified phosphatidyl-serine"¶ became ill.

To learn about the distribution of the "antigen," optic nerves, which are composed only of white matter, were procured post-mortem from 2 human beings, care being taken to avoid the perichiasmal tissue. One of 3 guinea pigs injected with an emulsion made from this tissue became paralyzed.

In summary, it would seem that the "anti-

[6] Morrison, L. R., *Arch. Neur. and Psych.*, 1947, **58**, 391.

[7] Stevenson, L. D., and Alvord, E. C., Jr., *Am. J. Med.*, 1947, **3**, 614.

‡ A preparation generously supplied by Dr. Jordi Folch-pi, McLean Hospital, Waverley, Mass., and said to contain practically all of the lipids present in whole brain and to be free of protein and carbohydrate.

[8] Page, I. H., *Chemistry of the Brain*, Charles C. Thomas, Springfield, 1937, p. 54.

[9] MacLean, H., and MacLean, I. S., *Lecithin and Allied Substances, the Lipins*, Longmans, Green & Co., Ltd., London, 1927, p. 125.

§ A light paraffin oil, obtained through the courtesy of Mr. K. L. Patterson, Stanco, Inc., New York City.

‖ An adsorption base said to be a mixture of oxycholesterine and cholesterines derived from lanolin (Pfaltz and Bauer, Inc., New York City).

¶ Also supplied by Dr. Folch-pi and described in *J. Biol. Chem.*, 1942, **146**, 35.

gen" responsible for the meningoencephalomyelitis is a phosphatide-like material present in the white matter of the central nervous system. Its properties serve to differentiate it from 4 of the 5 haptens thought to be present in brain.[7] "Neurokeratin"[10] has been eliminated by previous workers;[5] "protagon" and "sphingomyelin"[11] are included in the non-phosphatide fractions tested in the present experiments and found to be inactive; and the hapten thought to be present in gray matter[11,12] is obviously not necessary since it has now been found that white matter (optic nerve) contains the "antigen." Whether the "antigen" is the same as the "alcohol-soluble brain hapten"[11,12,13] must await further studies.

[10] Bailey, G. H., and Gardner, R. E., *Am. J. Hyg.*, 1942, **36**, 205.

[11] Schwab, E., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1936, **87**, 426.

[12] Reichner H., and Witebsky, E., *Ibid.*, 1934, **81**, 410.

[13] Rudy, H., *Biochem. Z.*, 1933, **267**, 77.

16341

Thiouracil and Mammary Growth.*†

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There are a number of reports in the literature which would appear to indicate that the mouse and the rat may respond differently, as regards mammary development and responsiveness, during experimentally induced hypothyroidism.

Mixner and Turner[1] have reported an increased mammary responsiveness of ovariectomized female mice to estrogen and progesterone treatment when thyroxin was simultaneously administered, while a decreased responsiveness followed thyroidectomy. Mixner[2] later found that thiouracil administration had an effect similar to thyroidectomy in decreasing mammary responsiveness. Morris *et al.*, found considerable mammary atrophy in virgin female mice fed thiourea[3] and thiouracil[4]. Gardner[5] observed mammary growth in male mice fed desiccated thyroid, but the effect was dependent upon intact testes.

From these reports it would appear that, in the mouse, experimentally induced mild hyperthyroidism is conducive to enhanced mammary responsiveness, while the opposite is true of hypothyroidism.

In the rat, however, Leonard and Reece,[6] and Smithcors and Leonard[7] have found that thyroidectomy of both male and female rats caused an enhanced development of the mammary gland. Thyroidectomy also increased the effectiveness of administered estrogen or testosterone propionate in stimulating mammary alveolar development. Chamorro[8,9] has also reported that thyroid-

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1097.

† The diethylstilbestrol and its dimethyl ether used in this investigation were generously provided by Merck and Co., Rahway, N.J., and the thiouracil by Lederle Lab., Pearl River, N.Y.

[1] Mixner, J. P., and Turner, C. W., *Endocrinology*, 1942, **31**, 345.

[2] Mixner, J. P., *J. Dairy Science*, 1947, **30**, 578.

[3] Morris, H. P., Dubnik, C. S., and Dalton, A. J., *J. Nat. Cancer Inst.*, 1946, **7**, 159.

[4] Morris, H. P., Dubnik, C. S., and Dalton, A. J., Exhibit Abstract, Fourth Internat. Cancer Res. Congress, Sept. 2-7, 1947, St. Louis.

[5] Gardner, W. U., *Endocrinology*, 1942, **31**, 124.

[6] Leonard, S. L., and Reece, R. P., PROC. SOC. EXP. BIOL. AND MED., 1941, **28**, 65.

[7] Smithcors, J. F., and Leonard, S. L., *Endocrinology*, 1942, **31**, 454.

[8] Chamorro, A., *C. R. Soc. Biol.*, 1946, **140**, 499.

ectomy of rats caused mammary hypertrophy and augmented the mammary stimulating effect of pregneninolone. Smithcors[10] has found that thiouracil treatment of rats did not of itself produce mammary alveolar development, but enhanced the mammary alveolar response to administered estrogen.

Unlike the situation in the mouse, these reports indicate that in the rat experimentally induced hypothyroidism is conducive to enhanced mammary gland growth and responsiveness.

With regard to hyperthyroidism in the rat, Weichert, Boyd, and Cohen[11] reported enhanced mammary development, but the effect was one of induced pseudopregnancy, and hence dependent upon intact ovaries.

Certain recent observations on thiouracil administration at this laboratory appear to fit into the above pattern.

Twenty-one young male albino rats were castrated and divided into three groups. Group 1 (6 rats) served as a control. About 10 days later Group 2 (8 rats) and Group 3 (7 rats) were started on daily injections of 10 μg of diethylstilbestrol in oil. Group 3 had 0.1% thiouracil added to its feed. At the end of 21 days of this treatment all groups were sacrificed. Body weight, pituitary lactogen content and thyroid weight changes in these animals have been reported previously in connection with another experiment.[12]

The mammary glands of the control group showed small to medium sized duct systems with little or no alveolar development. Group 2 showed an enhanced state of mammary development, with additional alveolar development in most animals. Group 3 showed a striking advancement of mammary development over the control group, and a marked improvement over Group 2. Extensive alveolar development was present with good duct extension.

The mouse experiment involved 15 intact albino males. Five served as controls. Four were maintained for 6 weeks on a grain ration containing 1.23 mg of dimethyl ether of diethylstilbestrol per kilo of feed. This level of estrogen was known to promote good mammary duct extension in male mice. Six were maintained for 6 weeks on the same level of estrogen with 0.2% thiouracil added to the feed after the first week.

The estrogen treated animals showed good duct extension as compared to the controls, with some alveolar development in one animal. Unlike the results of the rat experiment, no difference in the response to estrogen could be detected in the estrogen and thiouracil treated mice.

The conditions of the rat and mouse experiments are unfortunately different in several respects such as dosage, duration, and mode of administration. However, taken together with previous reports, the present results are indicative of a difference in the effect of thiouracil upon mammary responsiveness in the rat and mouse.

With regard to the rat, there has been reported an effect of hyperthyroidism and hypothyroidism on vaginal sensitivity to estrogen. The effect appears to parallel that on mammary sensitivity to estrogen. Van Horn[13] found that in 20 of 24 castrated female rats in a hyperthyroid condition, approximately 3 rat units of theelin were necessary to produce estrus. Langham and Gustavson[14] found the vaginal rat unit of estrone to be 1.33 μg for castrate controls and 2.5 μg for castrates receiving thyroxin injections. Conversely thyroparathyroidectomized castrates required only 0.86 μg. Thiourea administered in the drinking water caused a marked immediate decrease in vaginal sensitivity to estrone followed by a gradual increase, the rats becoming as sensitive after 56 days as thyroparathyroidectomized castrate rats.

It is possible that the same underlying mechanism is responsible for the effect of

9 Chamorro, A., *C. R. Soc. Biol.*, 1946, **140**, 721.

10 Smithcors, J. F., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 197.

11 Weichert, C. K., Boyd, R. W., and Cohen, R. S., *Anat. Rec.*, 1934, **61**, 21.

12 Meites, J., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 488.

13 Van Horn, W. M., *Endocrinology*, 1933, **17**, 152.

14 Langham, W., and Gustavson, R. G., *Fed. Proc.*, 1946, **5**, 143.

hypothyroidism on the observed variations in the vaginal and mammary sensitivity of rats to estrogen.

The seemingly anomalous situation of thiouracil enhancing mammary growth response in the rat may have an analogy in body growth also. Astwood[15] states that, although the prolonged administration of effective doses of thiouracil to rats causes an arrest of development and a retardation of growth, a small dose of thiouracil, given from the 21st day of life for a period of 9½ months, resulted in increased growth, including a gain in skeletal dimensions. The implication is that a mild degree of hypothyroidism is conducive to excessive growth in this species.

The authors are not familiar with any data indicating a similar relationship in the mouse.

Koger and Turner[16] have studied the effect of experimentally induced hyperthyroidism in 4 species, including the rat and mouse. In the mouse it was found that although excessive hyperthyroidism inhibited body growth, a mild degree of hyperthyroidism consistently accelerated the growth rate. In rats however, mild hyperthyroidism had no such desirable effect, with the possible exception of a few animals that showed limited acceleration of growth.

It would appear from such experiments that the rat is normally secreting a level of thyroxin close to the upper limit of tolerance, and may actually be benefitted in some respects by a slight reduction in the normal thyroxin secretion rate. If such is actually the case, this relationship would have wide implications in connection with the choice of experimental animals for certain types of investigation. It is known for instance that the normal rat is a poor animal for use in the assay of thyrotrophic hormone. Its thyroid gland is normally quite hyperplastic and relatively insensitive to further thyrotrophic stimulation.

That this relationship may not be true of all strains of rats is indicated by the work of Palmer *et al.*[17] These authors were able, by selection of the progeny of a single pair of rats, to produce two strains which differed appreciably in their efficiency of food utilization. This difference was accompanied by a difference in the basal metabolic rate and in the effect of administered thyroid on skeletal length and on efficiency of food utilization. This was interpreted by the authors as indicating a possible difference in the thyroid secretion rate of the two strains.

The inheritance of varying rates of thyroxin secretion may be responsible in part for the marked differences in the growth rate and body size of the Yale and Wistar strains of albino rats.[18]

Conclusion. It would appear that there exist differences in the reaction of the mouse and rat to experimentally induced hypothyroidism and hyperthyroidism, possibly dependent upon the relative thyroxin secretion rates of the two species.

15 Astwood, E. B., *N. Y. Acad. Med., Harvey Lectures,* 1944-45, **40**, 195.

16 Koger, M., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 377, 1943.

17 Palmer, L. S., Kennedy, C., Calverley, C. E., Lohn, C., and Weswig, P. H., *Minn. Agr. Exp. Sta. Tech. Bul.* 176, 1946.

18 Harned, B. K., and Cole, V. V., *Endocrinology,* 1939, **25**, 689.

16342

Effects of Electroshock Convulsions on Chronic Decorticated Cats.

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Masserman[1] has presented evidence that electroshock convulsions produce marked changes in the adaptive behavior of intact cats with experimental neuroses, comparable to the effects of electroshock therapy in man. In view of the evidence[2] that metrazol convulsions have excitant effects on autonomic centers of the hypothalamus, it was considered of value to investigate the possibility that electrically induced convulsions alter not only learned adaptive responses at the cortical level, but also non-learned, subcortically integrated responses which are closer to instinctive adaptive patterns. A study was therefore made of the effects of single and repeated electrically induced convulsions on the reactions of chronic decorticated cats to various stimuli, observations being limited to directly observable phenomena in the non-narcotized, non-curarized preparation.

In addition, this study afforded an opportunity to study the electroencephalographic patterns of electroshock convulsions in animals without neocortex. This was accomplished in terminal experiments on the decorticated cats after curarization.

Methods. Six cats were prepared by successive ablation of each cerebral hemisphere under nembutal anesthesia. Two operations were made, a week or more apart, under aseptic conditions without previous ligation of the carotid arteries. After recovery from anesthesia, all preparations exhibited the usual behavior and reactions of chronic decorticated cats and remained in good condition until terminal experiments were performed 29 to 147 days after completion of decortication. Observations were made on spontaneous behavior, posture, righting reflexes, licking reflexes, rectal temperature, pulse and respiratory rates, and sham rage responses to handling and to nociceptive stimulation. To evoke nociceptive responses, a device was employed which permitted the quick application of known pressures from 1.0 to 10.0 kg to a segment of the tail for one second. With this apparatus the pressure in kg per cm^2 necessary to elicit a given response is a function of the area over which the pressure is applied. Hence, in this paper, all references to "pressure" are expressed in kilograms, understood to be applied to the specific jaw area of the instrument (1 cm^2). Sham rage was also elicited in response to presumably non-nociceptive stimuli by holding the preparation by the loose skin around the back of the neck and alternately raising and lowering it rhythmically so that the hind footpads lightly touched the surface of the table on the downward movement. Control observations were made at intervals of 1 to 3 hours over a period of 8 hours on each of two successive days and before each experiment with electroshock convulsions.

Convulsions were produced by passing 200 to 450 milliamperes of 60 cycle A.C. for 0.2 to 0.3 sec. from vertex to palate. The electrode at the vertex consisted of a battery clip covered with saline paste attached to the scalp between the ears. The palate electrode consisted of a metal rod, one end of which was covered with gauze padding soaked in salt solution, and applied to the hard palate just behind the upper canine teeth. A commercial electroshock apparatus (Offner Type 735) was employed throughout. This device permits the delivery of a predetermined amperage regardless of variations in circuit resistance or size of the head. As will be shown below, the currents used appeared to exert no deleterious effects either physiologically or anatomically. From observations on conscious patients it is known that electroshock treatments are not painful.

[1] Masserman, J. H., and Jacques, M. G., *Am. J. Psychiat.*, 1947, **104**, 92.

[2] Gellhorn, E., *Am. J. Psychiat.*, 1941, **97**, 944.

Studies on the effects of electroshock convulsions were begun 7 to 37 days after completion of decortication in 5 of the preparations. The sixth was utilized only in the terminal experiment, 29 days after total decortication. After 48-hour observations on the effects of single electrically induced convulsions, 5 to 9 daily convulsions were induced, following which the preparations were observed during a 4- to 39-day period. One preparation was subjected to a second series of electroshock convulsions and observed for 7 days thereafter.

In terminal experiments carried out on 4 of the preparations, records were made of the electrical activity of the remaining brain immediately following passage of the current known previously to have induced convulsions regularly. This was accomplished by inserting, under ether, a tracheal cannula and screw electrodes into the calvarium and the body of the sphenoid bond (Fig. 1). The preparation was then immobilized by repeated intravenous injections of curare ("Intocostrin," 0.3 to 0.5 cc at ½ to ¾ hour intervals) and maintained on artificial respiration without anesthesia. Leads from the screw electrodes and one ear were connected to a capacity-resistance coupled amplifier-oscillograph system with optical recording on moving bromide paper. In all experiments the leads to the amplifiers were disconnected before and during the passage of the convulsing current and replaced as soon as possible (3 to 5 seconds) afterward, with simultaneous removal of the electroshock leads, to reduce amplifier blocking.

FIG. 1.
Cat No. 105 (chronic decorticate). X-ray of skull showing location of screw leads used for recording electrical activity of remaining brain in terminal experiments. Both screws are in the midline sagittal plane; the upper just penetrates the calvarium at the vertex and the lower extends to about 1 mm below the floor of the sella turcica in the body of the sphenoid bone.

After completion of these experiments the cats were sacrificed and the brain tissue was fixed *in situ* with formalin and removed for gross and microscopic study.*

Results. A. *Motor Pattern of Electrically Induced Convulsions.*

Immediately after electroshock the preparation assumed a posture of general semiflexion and for 15 to 20 seconds exhibited rapid rhythmic oscillatory movements of small amplitude involving the extremities, jaw and facial musculature. Frequently, several such paroxysms occurred, separated by quiescent periods lasting a few seconds. Finally, transient running movements of the hindlimbs appeared, after which all activity ceased for about 30 seconds. The preparation then righted itself or maintained the upright posture when so placed. During the seizure, apnea occurred, followed by transient hyperpnea. Occasionally, the preparation exhibited a burst of sham rage lasting 15 to 30 seconds, immediately after the seizure. One preparation circled for several minutes after each convulsion. With smaller currents greater variations were seen, such as sham rage without a preceding seizure or falling to one side in semiflexion without further motor activity. The motor pattern of the convulsions elicited by a given electroshock was remarkably constant for each preparation.

B. *Effects of Single and Repeated Electrically Induced Convulsions.*

Changes in temperature and pulse rate after single or repeated convulsions did not exceed spontaneous variations noted in control studies. No enduring impairment of righting re-

* The neuropathological examinations were made by Dr. I. Mark Scheinker, Assistant Professor of Neuropathology, University of Cincinnati College of Medicine.

flexes occurred after single or repeated seizures. In 4 of the preparations, vigorous licking movements of the tongue could be induced by tactile stimulation of the perineal region. The licking appeared to be directed to any object nearest the tongue, e.g., the forepaws, the experimenter's hand, etc. This response was abolished for from ½ to 3½ hours after each convulsion in 3 of the cats. In the fourth preparation, no change occurred. No additional or cumulative effects were noted after daily convulsions.

In control observations, the threshold for sham rage induced by rhythmically raising the cat from a table varied from one such maneuver in one cat to no reaction after 50 trials in another. The day-to-day variations were also considerable, except in the cat with the lowest threshold, which always exhibited sham rage after one maneuver. The rage pattern thus evoked consisted of springing or lunging, boxing and scratching movements of the forelimbs, extrusion of the claws, lashing of the tail, retraction of the angle of the mouth, dilation of the pupil, retraction of the nictitating membrane and piloerection. Vocalization, spitting or hissing did not occur. After each electrically induced convulsion, such rage reactions were either unaffected or increased as indicated by a reduction in the number of maneuvers needed to elicit the response compared with the control value for that day. After completion of a series of daily electroshock convulsions, there seemed to be some intensification of the rage response to handling, but this could not be confirmed by the threshold values which were not altered beyond the range of variation in the control studies.

In control observations the threshold for sham rage induced by graded pressure on the tail varied from 3.0 to 7.0 kg from cat to cat; in each preparation daily variations of about 1.0 kg were observed. The pattern of reaction to such stimuli was remarkably constant in each cat but varied in detail from one to the other. In general, the responses consisted of vocalization, spitting and hissing, struggling, lashing of the tail, mydriasis and retraction of the nictitating membrane. After each electrically induced convulsion, no reaction at all could be evoked even with maximum pressures of 10.0 kg, the limit of the instrument, for periods of from one to 2½ hours, and the thresholds in most instances did not return to the control level before 4 hours—*i.e.,* peak analgesic effects of over 50% to over 300% were observed. This was in marked contrast to the concomitant lowering of the rage threshold to handling described above. No significant changes occurred after completion of a series of daily electroshock convulsions.

C. *Electroencephalographic Patterns of Electroshock Convulsions.*

The electrical patterns of the seizures recorded in the terminal experiments varied in detail from one preparation to another, but the record illustrated in Fig. 2 A-C is fairly representative. After a single electroshock, several bursts of relatively high (75 to 100 microvolts) activity appeared, with frequencies varying from 2 to 21 per second. These paroxysms were separated by abruptly appearing silent intervals. In 3 of the experiments, a steady 15 to 18 per second rhythm appeared before cessation of the convulsive electrical discharge, which was followed by periodic isolated high voltage slow waves which were more prominent in tracings with a vertex lead. Such records could be obtained in each preparation several times by repeated electroshocks.

In places, a series of alternating fast and slow wave sequences was seen. Since in our laboratory typical "spike and dome" complexes have been observed in dogs following convulsant doses of morphine, we investigated the effects of this drug on the electrical patterns described above (Fig. 2 D-G). It will be noted that the slow and fast wave sequences after electroshock are more prominent in the morphinized preparation.

D. *Neuropathologic Findings.* On removal of the remaining brains after terminal experiments, the dead space left by removal of the cerebral hemispheres was found to be occupied by fibrous connective tissue which bound the dura to the underlying brain structures. Coronal sections revealed no evidence of neocortex; varying portions of the caudate

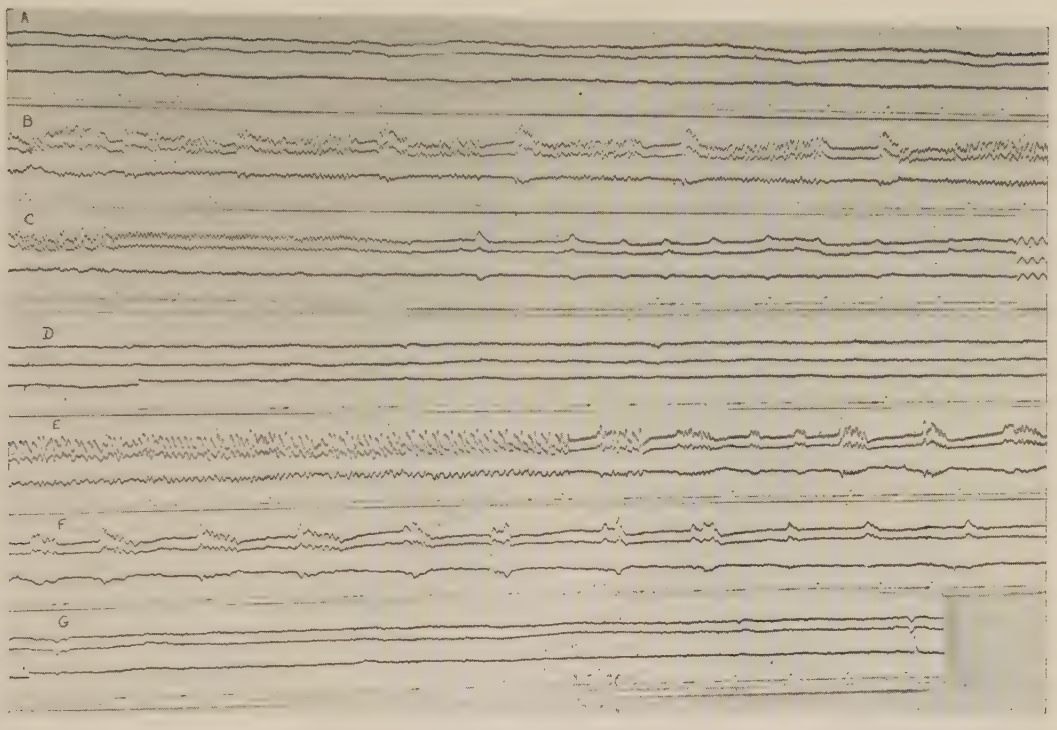

FIG. 2.

Cat No. 105 (chronic decorticate). Effects of electro-shock on electrical activity of remaining brain. Screw leads as in Fig. 1; clip lead on left ear. Preparation curarized and on artificial respiration. Time in seconds. Calibrations 23.8 microvolts peak-to-peak. In all records upper tracings are sphenoid to vertex, middle tracings sphenoid to ear, and lowest tracings vertex to ear. A, control. No significant electrical activity at this gain. B, 3 to 5 seconds after passing 450 milliamperes 60 cycle A.C. from vertex to palate for 0.3 sec. Note paroxysmal discharges of high voltage 6 to 21 per sec. waves originating in vicinity of the sphenoid lead. In places, a fast and slow wave occur in sequence, most often at the beginning of a paroxysm. C, continuation of preceding record. Note terminal regular 18 per sec. discharge of moderate voltage and random slow waves as record returns to control state. D, resting record 35 minutes after intravenous injection of morphine 20.0 mg per kg. No significant change from A. E, 3 to 5 sec. after electroshock as in B. Note paroxysmal discharges as in B, with greater tendency for fast and slow waves to occur in sequence. F and G, continuous with E. Note subsidence of electrical activity and return to control state.

nuclei, thalami and basal parts of the pyriform lobes were preserved. Microscopic study revealed evidence of degeneration with glial and connective tissue proliferation in the basal ganglia, especially in the periphery. In one specimen changes indicative of granular ependymitis were seen, and in another, evidence of mild subarachnoid hemorrhage. These changes did not differ in degree or kind from changes seen in the remaining brains of other chronic decorticated cats not subjected to electroshock or other convulsant agents in our laboratory.

Discussion. On comparison of our results with those of Masserman,[1] it is evident that, although transitory changes did occur in the licking and sham rage reactions of the former, recovery was rapid and no enduring effects comparable to those in intact neurotic animals were observed. It would seem, therefore, that the long-enduring effects brought about by electroshock in Masserman's neurotic cats were due to changes involving the cerebral cortex. This inference is supported by the observations on the electroencephalographic changes in intact cats subjected to repeated electroshock convulsions, reported by Rubinstein and Kurland.[3]

[3] Rubinstein, H. S., and Kurland, A. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 348.

The differences between the immediate effects of electroshock convulsions on sham rage reactions to nociceptive tail pressure and to handling are also of interest in connection with the report by Pisetsky[4] of the disappearance of painful phantom limbs after electric shock therapy in a patient, who, though no longer complaining of pain, still displayed considerable effect. The observations on decorticated cats suggest that such analgesic effects may be partly subcortical in origin. However, since no cumulative or long-enduring effects were observed in decorticated cats it is inferred that the permanent disappearance of phantom limb pain in Pisetsky's subject was probably due to effects involving the cortex.

The striking features of the electrical activity recorded from sphenoid leads in chronic decorticated cats after electroshock are the recurrent paroxysmal hypersynchronous discharges, suggestive of "status epilepticus." The mixture of fast and slow frequencies during such paroxysms resembles the pattern seen in the clonic phases of convulsions in man, while the terminal steady 15 to 18 per second discharge is more like that occurring in tonic seizures. Also noteworthy, especially in the morphinized preparations, are the rhythmic fast and slow wave sequences which resemble to some extent "petit mal" spike and dome complexes. However, the "spike" frequencies in the decorticated preparations were not faster than 24 per second, perhaps differing in this respect from cortical seizure discharges in man. Further studies are in progress to determine more precisely the relationship of the subcortical convulsive electrical discharges to those recorded from the cortex and to the motor seizure patterns.

[4] Pisetsky, J. E., *Am. J. Psychiat.*, 1946, **102**, 599.

Summary. (1) The effects of single and repeated convulsions induced by electroshock were observed in 6 chronic decorticated cats. (2) The seizures were characterized by a posture of general semiflexion with small amplitude rapid rhythmic movements of the limbs, jaws and facial musculature, interrupted by one or more short quiescent periods, terminating in running movements after which a more prolonged quiescent phase preceded recovery. Apnea occurred during the seizures and was followed by transitory hyperpnea. (3) Body temperature and pulse rate were not affected significantly. Righting reflexes returned a few minutes after each seizure. Licking reflexes were abolished for from ½ to 3½ hours after each convulsion in 3 preparations. Sham rage in response to a non-nociceptive stimulus was unaffected or enhanced temporarily after each electroshock convulsion. Sham rage responses (chiefly facio-vocal) to nociceptive pressure stimuli applied to the tail were markedly reduced or abolished for from 1 to 2½ hours after each electrically induced seizure. (4) No changes other than those noted after single electroshocks were noted after repeated electrically induced convulsions. (5) The electroencephalographic patterns of electroshock convulsions in the decorticated preparations were characterized by bursts of relatively high voltage 2 to 21 per second rhythms separated by short silent intervals, frequently terminating in a steady 15 to 18 per second discharge before cessation of electrical activity. Slow and fast wave sequences appeared at times during the paroxysmal discharges and were more prominent in previously morphinized preparations. (6) Gross and microscopic studies of the remaining brains after completion of the experiments revealed no changes which could be ascribed to the electric currents used to evoke convulsions.

16343

Cultivation of *Rickettsia prowazeki* in Dead Chick Embryos.

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The criterion commonly used for the determination of the state of life or death of an organism is the heartbeat. Chick embryos in which the heart has ceased to function must be regarded as dead, and this assumption is supported by the fact that such embryos are indeed incapable of further development.

However, an egg containing a dead embryo is not necessarily devoid of living cells. It is known that the cells of an organism may survive after death for varying periods, depending on the external condition and on the type of tissue from which the cells were derived. Individual cells in fully developed tissues generally survive no more than a few hours if they remain in contact with the dead organism. However, in an embryo, in which cells are probably less interdependent, conditions need not be, and indeed are not, the same.

It has been demonstrated by Bucciante[1] that dead chick embryos may contain living cells for as long as 31 days after cessation of the heart beat, if the egg be maintained at 20°C after the death of the embryo; survival times at lower temperatures were even longer, up to 47 days.

It occurred to us that eggs containing surviving rather than developing tissue might constitute a favorable medium for the cultivation of *Rickettsia prowazeki* and related organisms.

According to Zinsser and Schoenbach[2] the rickettsiae develop best in cells with a low metabolic rate, and such a state may be assumed to occur in cells which survive without development for extended periods. However, the question was whether the cells would survive sufficiently long at temperatures high enough for the development of rickettsiae.

Grodzinski,[3] who examined the survival at 38°C of aorta tissue from dead chick embryos, reported a maximum survival time of 3 hours, which is, of course, insufficient for the cultivation of rickettsiae. However, this author tested only one type of tissue and worked with relatively well developed embryos, the youngest being 7 days old.

We tried using embryos which had been killed by chilling, after only 3 days development, and found that living cells could still be demonstrated in such material after much longer periods, even if the eggs had been maintained during this time at 37°C.

The following two cases may serve as illustrations: An egg containing a 3-day-old embryo was chilled at 4°C for 24 hours, and, after storage for 4 days at room temperature, was incubated for 10 days at 37°C. When the egg was opened after this period it contained a dead embryo which had not developed beyond its third day, yet living cells, mostly of epithelial character, grew out when fragments of the embryo or of its membrane were cultured in plasma clots.

In another case living cells were demonstrated by the same technique in an embryo which had been kept after death at room temperature for 7 days, and then for 16 days at 37°C. Results were practically the same in cases in which the embryos died spontaneously during the first 3 days of incubation as in those in which they were killed by exposure to cold.

R. prowazeki was inoculated into the yolk sac of eggs containing dead embryos and these were incubated for varying periods of time at 37°C. As expected, the rickettsiae multiplied abundantly under these conditions. It

1 Bucciante, L., *Arch. exp. Zellforsch.*, 1931, **11**, 397.

2 Zinsser, H., and Schoenbach, E. B., *J. Exp. Med.*, 1937, **66**, 207.

3 Grodzinski, Z., *Arch. exp. Zellforsch.*, 1932, **12**, 587.

is difficult quantitatively to compare the growth of rickettsiae in dead embryos with that in living ones, as several factors obviously influence the final result. The maximum number of rickettsiae obtainable per egg is perhaps greater in eggs containing living embryos, since their yolk sacs are more extensive than those of eggs which have developed for only 3 days. However, the concentration of rickettsiae per cell in the infected cells of dead embryos may surpass that reached in living ones.

The growth period for rickettsiae in dead embryos may easily be extended to 16 days, or double the time customary in work with living embryos. Therefore even minute inocula have a chance to multiply significantly. This was determined in the following manner: 3-day-old dead embryos and 7-day-old living embryos were inoculated in parallel series with decreasing amounts of the same suspension of rickettsiae. Inocula too small to yield a positive smear after the usual 7-days' incubation in the living embryos yielded smears containing numerous rickettsiae after 14 days' incubation in the dead embryos.

Several advantages are inherent in our method. Eggs have to be incubated prior to use for only 3 days, and may then be stored for sometime at room temperature like ordinary culture media. This fact is especially convenient in field work, in which eggs may be inoculated on the spot and thereafter transported to the laboratory without particular regard to correct incubation temperature, humidity, or protection from vibration during transport, as is necessary when dealing with living eggs. Furthermore, this method is economical, since the percentage of eggs which have to be discarded on account of premature death of the embryo is, of course, considerably reduced.

Summary. 1. Chick embryos which were killed by chilling on the 3rd day of development and were thereafter maintained at 37°C for 16 days still contained living cells.

2. *Rickettsia prowazeki* multiplied abundantly in dead chick embryos which contained surviving cells.

3. The significance of these findings and some advantages inherent in this method are discussed.

16344

Sectioning Techniques for Electron Microscopy Using a Conventional Microtome.

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Sectioning biological material for use with the electron microscope poses special problems. Useful thicknesses can be measured only in fractions of a micron so that cutting becomes a major difficulty. Sections have to be examined *in vacuo,* so that the mode of drying is a matter for serious consideration.

The authors know of only 3 prior attempts to adapt more or less conventional histological techniques to the preparation of specimens for the electron microscope. Richards, Anderson and Hance[1] took embedded material and faced the block in a standard microtome. They then changed the angle of the block and cut wedges which they hoped would taper into the fractional micron range. To a certain extent they were successful, but apparently were unable to find a completely satisfactory solution to embedding and mounting problems. Von Ardenne[2] employed essentially the same principle. Sjöstrand[3] has also attempted

[1] Richards, G. A., Anderson, T. F., and Hance, R. T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 148.

to cut thin sections in an unspecified manner. Interest then shifted to the development of high speed microtomes by O'Brien and McKinley[4] and Fullam and Gessler.[5] Their cutting edges, moving with tremendous linear speeds, have cut sufficiently thin sections for effective use. But the design and construction of the high speed microtome is a complex engineering problem, and it is certain that it will always be a very costly instrument, not usually available.

The possibility that standard histological techniques could be adapted was first suggested to the authors by Dr. F. Kiss of Hungary. In conversations he stated that he had often cut fractional micron sections for use with the light microscope. He implied that the size of the block was a critical factor, and that the material should be doubly embedded in collodion and paraffin. It now appears that his claim was fully justified, for we have consistently been able to cut sections 0.2 microns in thickness.

A Spencer rotary microtome model No. 820 is used, but altered simply so that the unit of advance is approximately 1/10 of the calibrated value. To accomplish this the angle of lever action is reduced 90% with the attachment shown in Fig. 1 (which is readily removable for standard use).

Our technique has been developed using rat liver perfused with 2% osmic acid in the manner of Claude and Fullam[6] who have published micrographs of guinea pig liver sectioned with the high speed microtome. Small blocks of tissue (approximately one mm cubes) are run up through the alcohol series, into ether-alcohol, then into 3, 6, and finally 10% collodion (Mallinckrodt Parlodion) dissolved in ether-alcohol. The collodion is hardened in chloroform, and the blocks transferred to xylol by way of carbol-xylol. They are then infiltrated with 65°C paraffin. The blocks are finally mounted rigidly in the microtome after being pared down to present a face about one mm square.

To cut 0.2 micron sections it is hardly necessary to say that the knife must be very sharp, but proper stropping is quite adequate. The optimum adjustment of the knife tilt is extremely critical, and even though alignment marks are lined up on the holder, minor adjustments are still necessary every time the knife is moved or replaced.

The sections are cut at moderate speeds, perhaps slightly faster than would ordinarily be used. They tend to rumple somewhat as they are cut. But single sections or short series can be picked up with a fine brush and transferred to a standard specimen screen for the electron microscope. Then, working with a dissecting microscope, one or two corners are tacked down to the screen by pressure with a needle. Gentle teasing with either the brush hairs or with needles will take out the major wrinkles. Finally the section is flattened and given many points of contact with the screen by gently stroking it with the brush, or by rubbing with the blunt polished end of a glass rod. A flattened section on a screen can be seen in Fig. 2.

The final preparation for the electron microscope involves the removal of part or all of the embedding material, with the hazards attendant upon the final evaporation of the solvents. Three methods have been devised, each of which has certain advantages, so that it is worthwhile to consider all.

Method 1. The simplest preparation is to extract the paraffin but leave the collodion in place. This is done by dropping xylol repeatedly on the specimen screen and thus flushing it. It is finally air dried. The collodion which remains is not a serious handicap to observation, at least at a magnification of $\times$ 5,000, as it is in the form of a very fine mesh with low density. Since the specimen was impregnated with collodion first, presumably all parts of the section remain supported in place by the collodion during the drying, and in spite of the paraffin extraction.

2 von Ardenne, M., *Z. Wiss. Mikroskopie,* 1939, **56**, 8.

3 Sjöstrand, Fritiof, *Nature,* 1943, **151**, 725.

4 O'Brien, H. C., and McKinley, G. M., *Science,* 1943, **98**, 455.

5 Fullam, E. F., and Gessler, A. E., *Rev. Sci. Inst.,* 1946, **17**, 23.

6 Claude, Albert, and Fullam, E. F., *J. Exp. Med.,* 1946, **83**, 499.

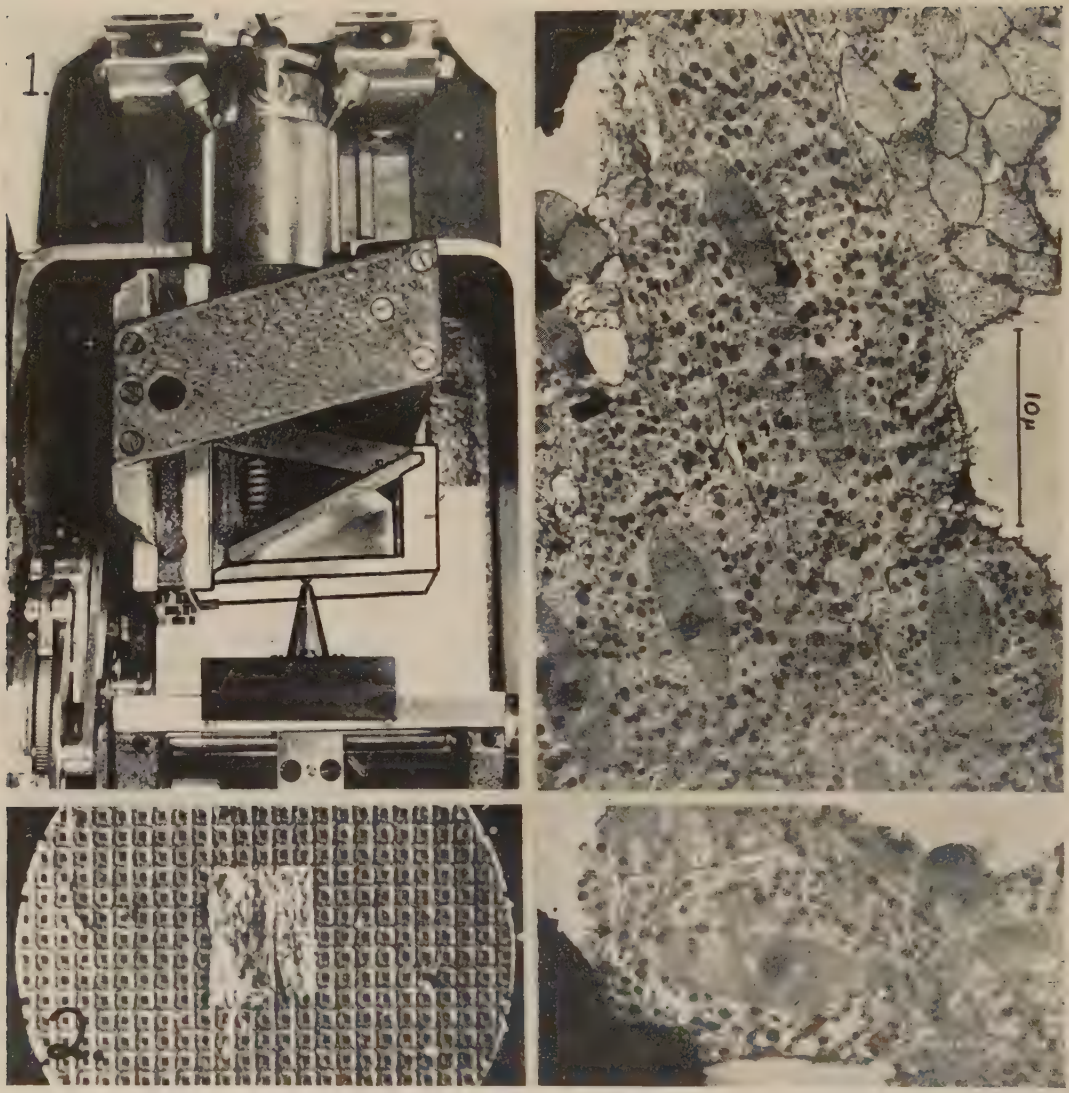
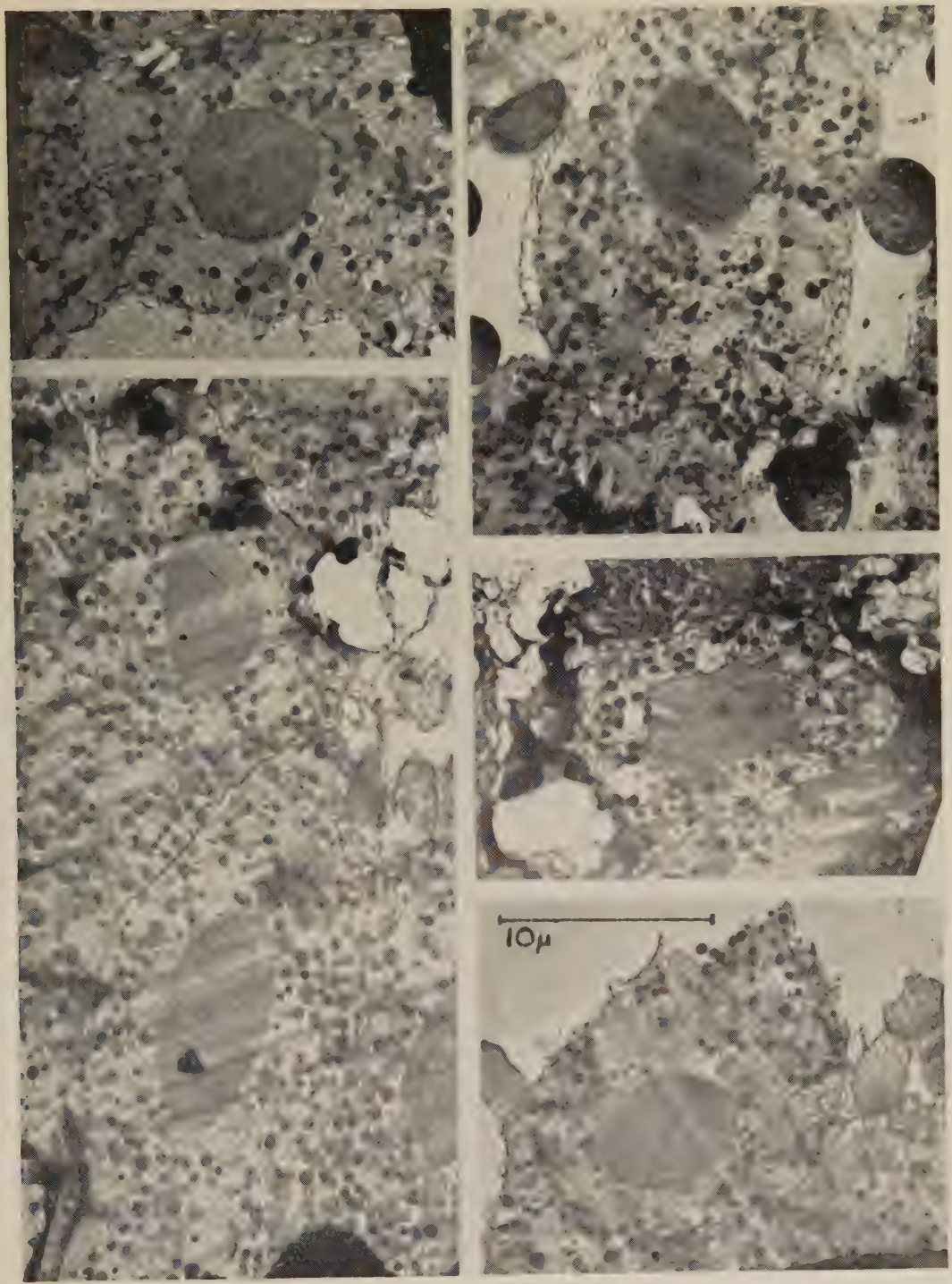


FIG. 1.
Vertical view of Spencer microtome modified by the outlined attachment to cut 0.1 micron.

FIG. 2.
Enlarged view of a 0.2-micron section mounted on a standard 200-mesh specimen screen. The remaining figures show electron micrographs of rat liver sections cut at 0.2 microns. Cell walls, nuclei, nucleoli, mitochondria, bile capillaries, and blood sinusoids are visible. Sections of red blood cells appear in some sinusoids, although most were removed by the perfusion technic employed in the initial fixation with osmic acid.

Such specimens are very strong and resistant to electron bombardment.

Method 2. If the embedding media are to be entirely extracted surface tension may have serious distorting effects during the final drying of the solvents. However, if benzene is used as the final solvent it can be frozen (5°C) and sublimed from the solid state thus minimizing the danger. Adherence of section to screen is none too good during this treatment. Best results are obtained by observing the following points. The screen, with the section tacked on, is inverted on a clean glass slide, and held down with a


10μ

needle. Amyl acetate is dropped on top and the screen flushed, thus removing the collodion. The section is very likely to become detached at this time, and surely will later if the screen is not now squeezed against the section once more. After rubbing the back of the screen with a blunt instrument the section sticks to the screen rather than to the glass, and the screen can be picked up and transferred to benzene which will remove the paraffin. Finally a drop of benzene surrounding the screen is frozen, and the benzene sublimed *in vacuo*. After such a total extraction the section is fragile and does not stand up well under the electron beam. This method is desirable particularly when the fine structure of a supporting medium might introduce uncertainty in the interpretation of the micrographs.

Method 3. In certain situations it presumably would be desirable to prepare serial sections. It is possible to do this, at least with short series, by varying the process as follows. Instead of tacking the sections to screens, they are flattened against a clean glass slide by teasing and brushing. Amyl acetate is flowed over them, followed by benzene, and finally amyl acetate once more. Then the slide is dipped in a 1% solution of collodion in amyl acetate and drained. The sections adhere to the glass throughout this process and are embedded in a thin film of collodion which is then dried. The slide is now dipped in distilled water thus floating the film off on the surface. Screens are then placed over the portions of the film containing sections, and are finally picked up and handled by usual techniques. Drying occurs only after the film is in place for support. A number of sections can be prepared simultaneously in this way. The paraffin is removed entirely, and the original network of collodion is replaced by a thin amorphous film that is none the less quite tough.

The question of artefacts introduced by the method remains to be considered. The knife edge, as it cuts, has little tendency to compress the section. Some distortion may result from drying, but in most parts of a section is scarcely noticeable. The sections are not entirely uniform in thickness, but the variations are estimated to be usually less than .05 micron. Due to the non-uniformity one can find local areas that are probably less than 0.1 micron thick. Lines resulting from the chatter of the knife edge are sometimes present, and easily recognized.

The principal artefacts are certainly those of the original fixation as Claude and Fullam[6] recognized in their work. In spite of very different treatments subsequent to fixation our micrographs are almost identical with theirs. Fixation artefacts become serious at magnifications above $\times$ 5000 when it can be seen that much of the fine structure consists of precipitated fibers. The full potentialities of section cutting for the electron microscope, therefore, will be realized only after the development of superior methods of initial treatment.

Summary. Conventional histological techniques have been modified so that is is possible consistently to cut 0.2 micron sections for use with the electron microscope. The material must be doubly embedded in strong collodion and hard paraffin. The face of the block to be cut must be small, and the tilt of the knife must be precisely adjusted.

Procedures have been developed for partly or wholly removing the embedding media, and mounting the sections for the electron microscope.

Micrographs of rat liver sections show that the principal artefacts are due to fixation rather than subsequent treatments.

16345

Hemolysis with Human Complement, Human Cells, and Tannic Acid: Application to Complement Fixation Test.*

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It has long been known that tannic acid has an effect on erythrocytes which is analogous to the action of amboceptor; small concentrations of this substance produce agglutination of the cells but no hemolysis, while the addition of complement results in hemolysis.[1,2] An investigation of this phenomenon was undertaken in this laboratory, as part of a study on the mechanism of action of complement. At first, considerable difficulty was encountered in demonstrating a consistent hemolytic effect with tannic acid and complement. It was found that the reaction was extremely sensitive to slight variations in the concentration of the sodium chloride solution employed as diluent for the reagents involved. When, however, the concentration of salt solution was fixed at 0.7%, rather than the usual 0.85%, it became possible to determine the optimal concentrations of tannic acid, complement and red cells for the reaction, with reproducible results. It was then learned that lysis of human erythrocytes could be brought about by fresh serum from the same or other individuals, in the presence of tannic acid. Moreover, accurate titrations of human complement could be made which yielded results comparable to those in the standard amboceptor-sheep cell system.

The present report deals with an application of the above findings to the complement fixation test, using human cells, human serum, and tannic acid as the indicator system. By this method, estimations of specific antibody for lymphocytic choriomeningitis virus, streptococcus MG polysaccharide, and Wassermann antigen were performed, with results similar to those obtained by the standard complement fixation test.

Materials and Methods. Diluent. Sodium chloride dissolved in distilled water to make a 0.7% solution was employed as diluent for all reagents used in the test, in place of the usual physiological saline. The importance of using this diluent will be demonstrated below.

Cells. Blood obtained from a normal human subject was added to oxalate crystals. The cells so obtained were washed 3 times in physiological saline solution, and then suspended in sufficient 0.7% saline to make a cell concentration of 1%.

Complement. Whole human blood was allowed to clot at room temperature and then centrifuged at 2,000 r.p.m. for 10 minutes. The serum so obtained was kept in an ice bath. Fresh lots of cells and complement were prepared each day.

Tannic Acid. A single lot of tannic acid‡ was used in all the tests to be described. A 1% solution of tannic acid in 0.7% saline was made each day as a stock solution, and 2-fold dilutions from this were tested for the optimal tannic acid concentration by the method described below. In practice, it was found that a dilution of 1:16, or a 0.06% solution, was invariably suitable for producing hemolysis with complement.

Antigen-Antibody Systems. Three systems were employed in the tests to be reported. These were: (1) *Lymphocytic choriomeningitis virus antigen and antibody.* The antigen

* This work was supported by a grant from the Life Insurance Medical Research Fund.

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1 Reiner, von L., and Fischer, O., *Z. f. Immunitätsforsch.*, 1929, **61**, 317.

2 Landsteiner, K., *The Specificity of Serological Reactions*, Harvard University Press, 1946.

‡ C. P. Crystalline Tannic Acid, lot No. 1, 1945, J. T. Baker Co., Phillipsburg, N.J.

consisted of guinea pig spleen soluble antigen, prepared by the method of Smadel, Baird and Wall.[3] The antiserum was hyperimmune guinea pig serum, prepared after the method of these authors. (2) *Streptococcus MG polysaccharide antigen and antibody.*§ The antigen consisted of a solution of purified polysaccharide from streptococcus MG,[4] diluted 1/50,000 in 0.7% saline. Rabbit antiserum against this material was used as antibody. (3) *Wassermann antigen and antibody.*‖ A standard preparation of Eagle beef-heart Wassermann antigen and several specimens of human luetic sera were used.

Each of the sera was heated for 30 minutes at 56°C before the test. Appropriate controls for the anticomplementary effect of both sera and antigens were included in each test.

Sensitization of Cells with Tannic Acid. Two parts of a 1% suspension of washed human red cells were mixed with one part of 0.06% tannic acid, in 0.7% saline. This mixture was prepared at least 15 minutes before being used, for reasons which will be described below.

Titration of Complement. Complement was titrated in the presence of the antigen used for the test. It was usually sufficient to set up 6 different amounts of complement, *e.g.* 0.07, 0.06, 0.05, 0.04, 0.03, and 0.02 cc of serum each contained in 0.2 cc of 0.7% saline. To each amount of complement were added 0.2 cc of the antigen, and 0.2 cc of 0.7% saline. These mixtures were then incubated at 37°C for 30 minutes, since this was the duration of fixation in all tests to be described. Following this period, 0.6 cc of the mixture of cells and tannic acid were added to each tube. The results were read after 30 minutes further incubation at 37°C. The endpoint, or the last tube in which complete lysis occurred, was selected as one unit. For the complement fixation test, 1.5 units were employed.

The Test. The following amounts of the various reagents were used: Complement—0.2 cc (containing 1.5 units). Antigen—0.2 cc. Serum—0.2 cc. Sensitized cells—0.6 cc, consisting of 0.4 cc of 1% cells and 0.2 cc of 0.06% tannic acid.

Antiserum, antigen, and complement were added to the tubes in the order named. The tubes were then placed in a 37°C water bath and incubated for 30 minutes. At this time, 0.6 cc of sensitized cells were added to each tube, and the tubes reincubated for 30 minutes. Readings were then made of the degree of hemolysis. Fixation of complement was interpreted as occurring in those tubes showing no lysis, or one-plus lysis on a scale of 4+.

Results. The effect of small variations in the concentration of sodium chloride on the hemolytic reaction with human cells, human complement, and tannic acid is shown in Table I. In the experiment illustrated here, titrations of a sample of human complement were made in the presence of 0.06% tannic acid and 1% red cells. In each titration a different concentration of sodium chloride was used as diluent for the reagents involved. It will be seen that the greatest complement activity was evident in the row in which 0.7% sodium chloride was used as diluent, while the reaction was almost completely inhibited in concentrations of 0.85 and 0.9% sodium chloride.

The optimal concentration of tannic acid was found to be between 0.06 and 0.03%. When stronger solutions of tannic acid were used, for example a 0.1% solution, hemolysis did not occur. Solutions of less than 0.01% were inactive.

One percent suspensions of red cells yielded more consistent and reproducible results than 2% suspensions. With higher concentrations of red cells, incomplete hemolysis usually occurred regardless of the amounts of tannic acid or complement.

It was of great importance to sensitize the red cells with tannic acid at least 5 minutes before adding them to complement. If cells

§ Streptococcus MG polysaccharide and antiserum were kindly supplied by Dr. Frank L. Horsfall, Jr.

‖ Wassermann antigen and luetic sera were kindly supplied by Dr. Thomas Farmer.

[3] Smadel, J. E., Baird, R. D., and Wall, M. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 71.

[4] Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., and Horsfall, F. L., Jr., *J. Clin. Inv.*, 1945, **24**, 227.

TABLE I.
Effect of Saline Concentration on Lysis of Human Red Cells by Human Complement and 0.06% Tannic Acid.

% of NaCl	Volume of Complement in 0.2 cc						Controls	
	.06	.05	.04	.03	.02	.01	.06 cc complement	.06% tannic acid
.60	++++*	++++	++++	+++	0	0	0	0
.65	++++	++++	++++	++++	0	0	0	0
.70	++++	++++	++++	++++	+++	0	0	0
.75	++++	++++	+++	++	0	0	0	0
.80	++++	++++	++	0	0	0	0	0
.85	++	+	0	0	0	0	0	0
.90	+	+	0	0	0	0	0	0

* ++++ Complete hemolysis.
\+ Slight hemolysis.
0 No hemolysis.

TABLE II.
Complement Fixation with Lymphocytic Choriomeningitis, Streptococcus MG Polysaccharide, and Wassermann Antigens and Antibodies, Using Human Complement, Human Red Cells, and Tannic Acid.

Serum	Antigen	Initial serum dilution					Antigen Controls
		1/40	1/80	1/160	1/320	1/640	
Anti-MG	MG*	0‡	0	0	0	++	++++
	LCM†	++++	++++	++++	++++	++++	++++
	NaCl	++++	++++	++++	++++	++++	++++
Anti-LCM	MG	++++	++++	++++	++++	++++	
	LCM	0	+	++	+++	++++	
	NaCl	++++	++++	++++	++++	++++	
		1/8	1/16	1/32	1/64	1/128	
Luetic	Wassermann	0	0	0	0	+	++++
	NaCl	++++	++++	++++	++++	++++	
Normal	Wassermann	++++	++++	++++	++++	++++	
	NaCl	++++	++++	++++	++++	++++	

* Streptococcal MG polysaccharide 1/50,000.
† Lymphocytic choriomeningitis virus soluble antigen, from guinea pig spleen.
‡ Symbols as indicated in Table I.

and tannic acid were added separately to complement, little or no hemolysis occurred. If tannic acid and complement were allowed to stand together for a few minutes before the addition of cells, no hemolysis occurred. When cells and tannic acid had been together for 15 minutes, they could be used for the test for a period of at least 8 hours without deterioration.

The results of complement fixation tests with the three antigen-antibody systems studied are shown in Table II. It will be seen that the anti-streptococcus MG polysaccharide serum fixed complement in a dilution of 1:320 with the polysaccharide antigen, and showed no fixation with the unrelated lymphocytic-choriomeningitis antigen. The antiserum for the latter antigen, on the other hand, fixed complement in a dilution of 1:80 and showed no fixation with the MG antigen. Normal rabbit serum showed no fixation with either antigen. The human luetic serum fixed complement with Wassermann antigen in a dilution of 1:128, while a normal human serum showed no fixation. Consistent results in the Wassermann test were more difficult to obtain than in the other antigen-antibody reactions described, because of the frequent appearance of an anticomplementary effect in Wassermann antigen with tannic acid-sensitized cells.

Each of the above sera and antigens were tested in 30-minute complement fixation tests by the usual method, using guinea pig complement, sheep cells, and rabbit amboceptor. The serum titers which were obtained

were substantially the same as those shown in Table II.

Comment. The observation of hemolysis of human cells by complement from the same individual, and the various conditions under which this reaction occurs, are of theoretical interest and suggest possible new approaches to the problem of complement's role in hemolysis. The complement fixation test itself may prove to be of practical value under some circumstances. There are certain advantages in a serological test which involves an homologous hemolytic system, instead of the 3 or more mammalian species which are represented in the usual complement fixation test. Moreover, the method makes it possible to do complement fixation tests under conditions where one or another of the usual reagents cannot be obtained.

There are certain limitations to the use of this method which should be pointed out. In Wassermann tests, the frequent finding of an anticomplementary effect of antigen presents a technical disadvantage. Moreover, the test is not feasible with antigens for which many human sera possess antibodies in low titer, such as influenza virus in allantoic fluid, since the complement employed for the test may be partially fixed due to the presence of antibody in the same serum. For the same reason, it is probable that certain tissue antigens, with which human sera may react non-specifically in low dilution cannot be tested by this method. However, in those systems which lend themselves to the homologous test, the results appear to be clear-cut and are reproducible.

Summary. Human erythrocytes undergo lysis in the presence of tannic acid and human complement, even when cells and complement are obtained from the same blood sample. This reaction is dependent upon an optimal concentration of 0.7% sodium chloride, and an optimal concentration of between 0.06 and 0.03% tannic acid. When these substances are present in proper concentration, human complement can be titrated with reproducible results.

Specific fixation of human complement by three separate antigen-antibody systems has been demonstrated, employing homologous erythrocytes sensitized by tannic acid. Antibody titers determined by this method were comparable to those obtained in the standard complement fixation test.

16346

Effect of Chloromycetin on Experimental Infection with Psittacosis and Lymphogranuloma Venereum Viruses.

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The results of the search for chemical and antibiotic substances which might be effective in the treatment of infections of viral and rickettsial origin have been reviewed recently by several groups of workers.[1-5] The contribution of Hamre and Rake[5] is of especial interest as regards the current subject since it deals particularly with viruses of the psittacosis-lymphogranuloma group. The pres-

[1] Andrewes, C. H., King, H., and van den Ende, M., *J. Path. and Bact.*, 1943, **55**, 173.

[2] Kramer, S. D., Geer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

[3] Jones, H., Rake, G., and Stearns, B., *J. Infect. Dis.*, 1945, **76**, 55.

[4] Cutting, W. C., Dreisbach, R. H., Halpern, R. M., Irwin, E. A., Jenkins, D. W., Proescher, F., and Tripi, H. B., *J. Immunol.*, 1947, **57**, 379.

[5] Hamre, D., and Rake, G., *J. Infect. Dis.*, 1947, **81**, 175.

ent paper amplifies our earlier report[6] on the use of Chloromycetin[7] in experimental infections with psittacosis virus and summarizes our observations on the treatment of embryonated eggs and mice infected with the virus of lymphogranuloma venereum.

Materials and Methods. Virus material. The 6-BC and P-4 strains of psittacosis were employed in this work. The former, isolated in 1941 by Dr. K. F. Meyer from a parakeet, was obtained from Dr. E. Lennette as lyophilized yolk sac material; it is well adapted to growth in embryonated eggs and is lethal for mice when inoculated by either the intracerebral or the intraperitoneal route. The latter, isolated in 1942 from a sick pigeon,[8] also grows profusely in the yolk sacs of embryonated eggs but is lethal for mice only when inoculated intracerebrally. The L. A. strain of lymphogranuloma venereum used in the present experiments was obtained in 1943 from the inguinal gland of a patient;[9] it has been maintained since in this laboratory by passage in the yolk sacs of embryonated eggs or brains of mice.

Preparations of the 3 agents used in the present work consisted of 20% suspensions of infected yolk sac tissue triturated in sterile Difco skimmed milk media pH 7.2; these were cleared of large particles by light centrifugation and stored in 2.0 ml amounts in rubber stoppered tubes at −70°C. The infectivity of each of the stored suspensions was determined by titration in embryonated eggs inoculated by the yolk sac route. In addition, each suspension was titrated intracerebrally in mice, and the preparation containing the 6-BC strain was also titrated intraperitoneally in mice. Titers were calculated by the 50% end point method.[10]

Chemotherapy experiments in eggs. The procedure developed in this laboratory for testing the efficacy of drugs on the growth of rickettsial organisms in embryonated eggs[11] was followed in the current studies. In this technique, groups of 24 embryonated eggs which had been incubated for 7 days were inoculated by the yolk sac route with 0.1 or 0.2 ml of a solution containing the desired amount of drug; the control group received 0.2 ml of buffered saline. Then all the embryonated eggs were again injected into their yolk sacs with 0.2 ml of that dilution of standard virus suspension which would be expected to kill the majority of the embryos in the control group in 4 to 5 days. Usually 30 to 45 minutes elapsed from the time an embryo was treated with drug until it received the infectious inoculum. In certain tests therapy was delayed for 24 to 48 hours after infection. Eggs were kept from the time of setting and throughout the experiment in an incubator regulated to 37°C. Following inoculation they were candled daily and the time of death of the embryos recorded. Deaths occurring in the first 2 days were not counted in the results. The mean day of death of embryos in each group was calculated. An estimate of the efficacy of the drug was obtained by subtracting the mean value of the control group from that of the treated group; this gave a figure which was taken as the average prolongation of life of the treated eggs. The methods for statistical analysis of the present data were the same as those used previously.[11] Dr. Ross L. Gauld performed these calculations for us.

Chemotherapy experiments in mice. Swiss mice of the Bagg strain, weighing 14-18 g were used in the present experiments. Mice infected by the intracerebral or intraperitoneal route received 0.03 ml or 0.2 ml, respectively, of an appropriate dilution of standard frozen virus suspension. Chloromycetin was administered to mice by mouth or injected intra-

[6] Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

[7] Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

[8] Smadel, J. E., Wall, M. J., and Gregg, A., *J. Exp. Med.*, 1943, **78**, 189.

[9] Zarafonetis, C. J. D., *New England J. Med.*, 1944, **230**, 567.

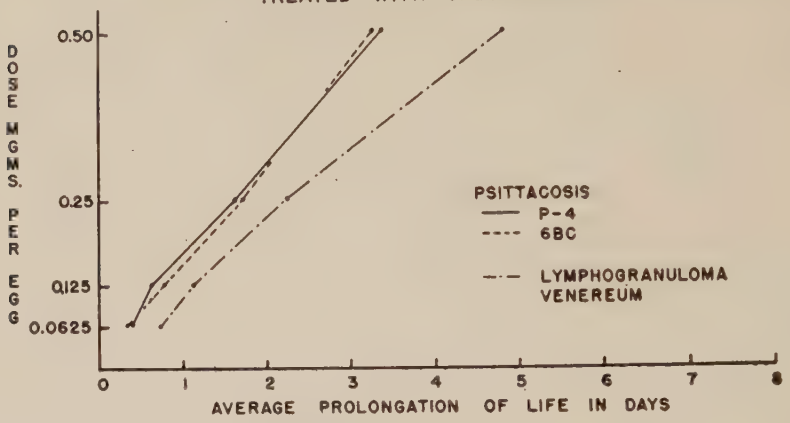
[10] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

[11] Smadel, J. E., Jackson, E. B., and Gauld, R. L., *J. Immunol.*, 1947, **57**, 273.

[12] Early, R. L., and Morgan, H. R., *J. Immunol.*, 1946, **53**, 251.

FIG. 1

PROLONGATION OF LIFE OF INFECTED EMBRYONATED EGGS TREATED WITH CHLOROMYCETIN



peritoneally. When the former route was used, sufficient drug was dissolved in distilled water so that each 1.0 ml of solution contained ½ the desired daily dose for a mouse. Daily at 5:00 p. m. each group of 10 mice was offered 20 ml of such solutions in a drinking bottle. This amount was usually consumed by morning unless the mice were moribund. During the day the animals were offered tap water. For therapy by the intraperitoneal route sufficient drug was dissolved in physiological saline solution so that each 1.0 ml contained the desired daily dose; this was given each morning at 9:00 a. m.

The lethal titers of the virus suspensions were calculated by the 50% end point method[10] for each set of control mice, and where feasible, for the groups of drug-treated mice.

Results. Prolongation of life of infected embryos treated with varying doses of drug and at different times. Fig. 1 summarizes experiments in which doses of drug varying from 0.0625 mg to 0.5 mg were injected into the yolk sac just prior to infection with several hundred M.L.D.'s of one of the 3 agents. In this figure the average prolongation of life of the treated groups expressed in days is plotted against the amounts of drug given. The values for prolongation of life obtained in tests with doses of 0.0625 mg and above were statistically significant. It is apparent that a direct relationship exists between the amounts of drug employed and the prolongation of life of the treated embryos. Furthermore, the results obtained with the 2 strains of psittacosis virus are essentially identical. In this method of testing, lymphogranuloma virus would appear to be slightly more susceptible to chemoprophylaxis with the drug than psittacosis virus.

Chloromycetin has a chemotherapeutic effect in infected embryos even when treatment is delayed for 24 or 48 hours. The results obtained in tests with the P-4 strain of psittacosis are illustrated in Table I; these are typical of the data obtained with each of the 3 agents. The prolongation of life in the group treated 24 hours after infection was, in each instance, slightly greater than that obtained when treatment was given ½ hour before infection. While the values for these differences in some of the 5 experiments with the 3 agents were such as might have occurred by chance, the fact that they all trend in one direction is significant. In the experiment illustrated, and in those with the other strains, the effect induced by treatment given 48 hours after infection approached that recorded for the group treated just prior to infection. In another experiment embryos were infected with approximately 1,000,000 M.L.D.'s of the 6-BC virus. The average day of death in the control group in this test was 3.1 days. Even when injection of the drug was delayed for 48 hours there was still a significant prolongation of life of

TABLE I.
Chloromycetin in Experimental Psittacosis in Eggs (Infecting Dose 500 MLD's of P-4 Strain).

No. of eggs in group	Drug treatment: mg/egg	Drug treatment: Time given (in hours)	Mean day of death	Prolongation of life (days)
24	None		4.83	
24	0.5	½ Pre	8.13	3.30
24	0.5	24 Post	8.77	3.94
24	0.5	48 Post	7.64	2.81

TABLE II.
Chloromycetin in Experimental Psittacosis in Mice (Infecting Dose 80 MLD's of 6-BC Strain Intraperitoneally).

Route	Drug treatment: mg/day mouse	Drug treatment: Day begun	Dilution of infectious inoculum: 10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
	None		10/10	7/10	8/10	6/10	2/10	0/10
I.P.	2.5	1 Post		1/10				
	2.5	3 "		1/10				
	2.5	6 "		1/10				
	1.5	1 "		0/10				
	1.5	3 "		2/10				
	1.5	6 "		1/10				
	0.75	1 "		0/10				
	0.75	3 "		4/10				
	0.75	6 "		7/10				
Fed	5.0	1 Pre		0/10				

TABLE III.
Chloromycetin in Mice Infected by Different Routes with 6-BC Strain of Psittacosis.

Exp.	Drug treatment: Route	Drug treatment: mg/day mouse	Drug treatment: Day begun	Infectious inoculum: Route	Dilution: 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Titer
		None		I.P. 0.2 cc	10/10	10/10	10/10	6/8	8/10	6/10	5/10	0/10	$10^{-8.3}$
1a	I.P.	2.5	1 post		3/10	0/9	2/9	0/8	0/9				$<10^{-3}$
	Fed	5.0	1 pre		5/10	2/10	2/10	0/8	0/10				$10^{-3.4}$
		None		I.C. 0.03 cc		10/10	10/10	10/10	10/10	10/10	5/10	0/10	$10^{-9.0}$
1b	I.P.	2.5	3 hours pre					10/10	10/10	10/10	4/10		$10^{-8.8}$
	Fed	5.0	1 pre					10/10	9/10	9/10	2/10		$10^{-8.5}$

the treated embryos, *i.e.*, 1.5 days.

Effect of Chloromycetin in mice infected by different routes. Mice infected by the intraperitoneal route with approximately 80 M.L.D.'s of the 6-BC strain of psittacosis were treated with Chloromycetin by the intraperitoneal or oral routes. Data from such an experiment are illustrated in Table II. In this and subsequent experiments the antibiotic was given to the mice in each test group from the time indicated until death occurred or until the 12th day after inoculation of the infectious material; treated and control mice were observed until the 21st day when the experiment was terminated. Seven of the 10 control mice which received the same concentration of challenge virus used to infect the treated mice died between the 6th and 10th

days. It is apparent from the tabular data that the daily intraperitoneal administration of 1.5 mg of Chloromycetin beginning 6 days after infection was effective since only one of the 10 mice in the group died. Half this amount of drug was ineffective when treatment was begun on the sixth day but did protect the entire group when given from the first day after infection. Finally, the data in Table II show that all mice which received approximately 5.0 mg of drug per day by mouth survived the infection.

A series of experiments was next performed in which mice were infected by the intracerebral route with the P-4 strain of psittacosis or the L. A. strain of lymphogranuloma venereum virus and treated with Chloromycetin either by mouth or by intraperitoneal injection. In none of these experiments was a beneficial chemotherapeutic effect demonstrated. The question then arose as to whether Chloromycetin would under any circumstance protect against intracerebral infection with a member of the psittacosis-lymphogranuloma group. The 6-BC strain of psittacosis was chosen for the next experiment because it is lethal when given either intraperitoneally or intracerebrally; this is in contrast to the P-4 strain of psittacosis and the lymphogranuloma virus which are lethal only when given by the intracerebral route.

In the experiment summarized in Table III, groups of mice were injected by one of these 2 routes with serial 10-fold dilutions of the 6-BC suspension and treated with Chloromycetin by daily injection or by feeding. It is apparent from the data presented in this table that treated mice survived inoculation with enormous amounts of virus injected by the intraperitoneal route but displayed no resistance to intracerebral inoculation of the virus.

Effect of Chloromycetin on the virus in vitro and in vivo. Chloromycetin possesses little or no direct virucidal action against the agent of psittacosis. This was demonstrated in an experiment in which 2 portions of a 10^{-5} dilution of a standard suspension of 6-BC strain were prepared; one contained the virus in ordinary milk diluent whereas in the other the virus was suspended in milk media in which was dissolved 2.5 mg of drug per ml. These 2 portions were held at room temperature for 2 hours and then titrated in embryonated eggs. The lethal titers were $10^{-9.6}$ and $10^{-10.3}$, respectively. Furthermore, the average day of death of the embryos inoculated with dilutions of the control and drug treated suspensions of virus were essentially the same. As was to be expected the lives of the embryos which received the original virus suspension containing 2.5 mg of drug per ml were prolonged.

In the present experiments none of the embryonated eggs inoculated with even a few hundred M.L.D.'s of virus survived to the time for hatching even when treated with 0.5 mg of drug. Yolk sac tissues from moribund treated eggs were as rich in elementary bodies as were those from controls.

In the experiment summarized in Table II, practically all of the mice which survived when treatment was begun later than the first day after infection developed obvious signs of disease. Those least affected showed ascites but otherwise appeared fairly healthy. No apparent illness was noted among the mice given the drug by mouth beginning the day before infection. In this experiment the spleens from 2 animals of each treated group in which all mice survived were tested for virus by passage to normal mice; each suspension of splenic tissue contained active virus. Furthermore, the remaining animals in these groups resisted an intraperitoneal challenge inoculation containing several hundred M.L.D.'s of the 6-BC strain. Thus, infection was not prevented in mice treated prophylactically nor were the tissues freed of virus when the drug was used for chemotherapy.

Direct evidence that Chloromycetin has a suppressive effect on the growth of virus in treated mice was obtained in the experiment summarized in Table III. Two mice from each of the groups which received a 10^{-6} dilution of virus were exsanguinated on the sixth day, care being taken to obtain the blood without contamination from the peritoneal cavity. At this time the treated mice looked healthy but the controls were sick. The

infective titers of the pooled hearts' bloods determined in mice were as follows: controls, $10^{-2.5}$; group treated with injections of 2.5 mg of drug, $10^{-1.3}$; group fed 5 mg of drug, less than 10^{-1} (1 of 5 mice receiving a 10^{-1} dilution of blood succumbed).

Discussion. Penicillin and drugs of the sulfonamide series have a therapeutic effect on experimental infections caused by a number of agents of the psittacosis-lymphogranuloma group. Furthermore, these substances have been employed in the treatment of patients with lymphogranuloma venereum or psittacosis (see [5]). The present data obtained in infected embryonated eggs and mice treated with Chloromycetin suggest that the new antibiotic possesses about the same degree of activity as that displayed by penicillin and sulfadiazine in similar types of studies.[5,12] It is of interest that none of the drugs are really effective in treatment of mice infected by the intracerebral route with these agents.

Conclusion. Chloromycetin possesses considerable therapeutic activity in embryonated eggs and mice infected with the viruses of psittacosis or lymphogranuloma venereum. This activity is comparable in amount to that demonstrated by others for sulfadiazine and penicillin tested under similar conditions.

16347 P

Inhibition of Multiplication of Influenza Virus by Tannic Acid.*

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Recently it has been reported[1-8] that various substances inhibit multiplication of one or more viruses of the mumps-influenza group as well as the pneumonia virus of mice. In some instances these substances inhibit both virus multiplication and virus hemagglutination. Moreover, of considerable interest is the fact that some of these substances are themselves capable of agglutinating erythrocytes. For the most part, when tested *in vitro* they exert little or no direct effect upon the viruses against which they are active *in vivo*. With the exception of the enzyme described by Burnet,[8] which apparently acts upon the receptor substance of the cell, the mode of inhibitory action and the relationship of inhibition of hemagglutination to that of multiplication are obscure.

The fact that tannin agglutinates erythrocytes has been known for some time.[9] During the course of studies on hemagglutination it was observed that tannic acid in concentrations as low as 45 μg per cc agglutinates chicken erythrocytes. Subsequent investigation showed that smaller amounts of tannic acid actually inhibit the agglutination of chicken erythrocytes by influenza A virus. Furthermore, tannic acid inhibits the multiplication of influenza A virus *in vivo* and inactivates the virus *in vitro*.

* Aided by a grant from the United States Public Health Service.

1 Wheeler, A. H., and Nungester, W. J., *Science*, 1944, **100**, 523.

2 Green, R. H., Rasmussen, A. F., Jr., and Smadel, J. E., *Pub. Health Rep., U. S. P. H. S.*, 1946, **61**, 1401.

3 Horsfall, F. L., Jr., and McCarty, M., *J. Exp. Med.*, 1947, **85**, 623.

4 Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.

5 Liebmann, A. J., Perlstein, D., and Snyder, G. A., *J. Bact.*, 1947, **54**, 63.

6 Rubin, B. A., and Giarman, N. J., *Yale J. Biol. and Med.*, 1947, **19**, 1017.

7 Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. ANL MED., 1947, **66**, 99.

8 Burnet, F. M., *The Lancet*, 1948, **254**, 7.

9 Freund, J., *J. Immunol.*, 1931, **21**, 127.

Methods. The tannic acid employed was a commercial sample. The PR8 strain of influenza A virus was used exclusively. Allantoic fluid stored in a CO_2 chest was used as a source of virus. With minor differences, tests for inhibition were carried out as previously described.[4]

Experimental. Tannic acid was dissolved in water, usually in a concentration of 2 mg per cc, neutralized with sodium hydroxide, autoclaved and again adjusted to pH 7. 0.25 cc amounts of serial 2-fold dilutions of such solutions were mixed with 0.25 cc amounts of saline containing 8 to 16 hemagglutinating units of influenza A virus. After an interval of 5 to 15 minutes 0.25 cc of a 1% suspension of chicken erythrocytes was added to each tube. Tannic acid, in final concentration of from 5 to 20 μg per cc, inhibits hemagglutination.

Varying amounts of tannic acid were injected into the chorio-allantoic sacs of 10-day-old embryonated hens' eggs and after an interval of one-half hour 10 to 100 ID_{50} of influenza A virus were inoculated by the same route. As a rule 0.5 cc containing one mg of tannic acid was injected. This amount of tannic acid inhibits the multiplication of virus to such an extent that the allantoic fluids from eggs so treated do not contain sufficient virus to produce hemagglutination. One mg of tannic acid injected into the allantoic sac at intervals up to 6 hours preceding the inoculation of virus as well as at intervals up to one hour following the inoculation of virus also causes inhibition of multiplication.

Allantoic fluid having an ID_{50} titer of 10^{-7} was diluted 1-40 with saline and a portion of this was mixed with an equal volume of saline containing 0.2 mg of tannic acid per cc; as a control, another portion of the same allantoic fluid was mixed with an equal volume of saline. After standing at 25°C for 15 minutes tests for infectivity were done by making serial 10-fold dilutions of each mixture and injecting aliquots of the various dilutions into the allantoic sacs of 10-day eggs. In such experiments tannic acid reduces the titer of virus by at least 3 logs.

Comment. The chemical effects of tannic acid on tissues are well known and have been summarized by Olitsky.[10] However, there is little information available concerning its effect on viruses. Olitsky *et al.* showed[10,11] that tannic acid instilled intranasally was able to prevent infection with the viruses of equine encephalomyelitis and poliomyelitis subsequently administered by the intranasal route. In this instance the action of tannic acid was said to be exerted on the nasal mucosa of the host rather than on the virus itself. The fact that tannic acid combines with proteins to form a material which shows a high degree of resistance to the destructive action of enzymes[10] provides interesting ground for speculation as to the mechanism of its inhibitory action. In the experiments reported, however, it would appear that at least part of the inhibitory effect of tannic acid is dependent on a direct action upon the virus.

Summary. Tannic acid inhibits both hemagglutination and multiplication of influenza A virus. *In vitro,* the virus is inactivated by tannic acid.

It is a pleasure to acknowledge the technical assistance of Miss Ann Holloway.

[10] Olitsky, P. K., and Cox, H. R., *Science,* 1934, **80**, 566.

[11] Olitsky, P. K., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 532.

16348

Lack of Action of Influenza Virus upon Mucin of Human or Swine Origin.*

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Francis[1] reported that normal serum in high dilution could inhibit hemagglutination by heated influenza virus. Burnet, McCrea, and Anderson[2,3] showed that (a) receptor destroying enzyme (RDE) of *Vibrio comma* and unheated influenza virus could destroy this inhibitor in normal serum, (b) a dilute solution of mucin derived from a pseudomucinous ovarian cyst and purified blood group substance A or O of human origin could inhibit hemagglutination by heated LEE influenza virus, and (c) RDE and unheated LEE virus could destroy this activity of mucin. The present report concerns the activity of influenza virus in the presence of purified preparations of human salivary mucin and gastric mucin of the hog.

The virus preparations were in the form of freshly harvested allantoic fluids of high titer derived from the infected chick embryo. The effect of various concentrations of human salivary mucin on 4 or 5 agglutinating doses of virus is recorded in Table I. The results show that as much as 2.5 mg/ml of mucin did not inhibit hemagglutination by heated or unheated influenza virus. No inhibition of hemagglutination was noted when gastric mucin of the hog was tested under the same conditions using swine influenza virus as well as the PR8 and LEE strains.

Since the salivary and gastric mucins did not inhibit hamagglutination, the technic used by Burnet, McCrea, and Anderson[3] could not be employed to determine whether or not influenza virus had any effect on mucin. However, an alternate technic utilizing the photoelectric colorimeter as a measure of turbidity was employed. In this type of experiment a small quantity of mucin in buffered saline was added to freshly harvested influenza virus. The results show that there was no decrease in the turbidity produced by mucin as a result of viral action (Table II). In addition the effect of the PR8 virus on both human salivary mucin and hog gastric mucin was studied to determine whether the virus could modify the conductivity or viscosity of mucin. In the conductivity test, there was no change in conductivity when 20 ml of PR8 virus with a final agglutinating titer of 1 to 40 acted on a solution of hog mucin or human salivary mucin containing 0.2 mg/ml of mucin in the presence of .0067M phosphate buffer at pH 7.4 at 25°C for 105 minutes and 204 minutes respectively. Using the Ostwald viscometer no alteration in viscosity of mucin was detectable in the presence of PR8 virus with a final agglutinating titer of 1 to 25 in .067M phosphate buffer at pH 7.4 when the virus acted on a 1% concentration of hog mucin and 0.25% concentration of human salivary mucin for 540 and 1017 minutes at 25°C respectively. These results are not surprising in view of the fact that Burnet, McCrea, and Anderson[2] have shown that the serological specificity of mucin is not destroyed by virus or by RDE. However, the clot or pellicle formed when a drop of the human salivary mucin or the gastric mucin was deposited in acid alcohol was dissolved by a drop of RDE thus indicating that these mucins were suitable substrates for RDE.

Two types of experiment were carried out to determine whether or not salivary or gastric mucin could inhibit the multiplication of in-

* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D.C.

† Fellow in the Medical Sciences of the National Research Council.

1 Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

2 Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160**, 404.

3 Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Austral. J. Exp. Biol.*, in press.

TABLE I.
Effect of Salivary Mucin on Hemagglutination by Viruses.

Virus	AD* of virus	Mucin mg/ml	Hemagglutination Dilution of mucin 4	8	16	32	64	128	256	512	1024	Mucin control
PR8	4	2.5	+	+	+	+	+	+	+	+	+	—
PR8 heated 56°C/10	4	2.5	+	+	+	+	+	+	+	+	+	—
SW	5	10	+	+	+	+	+	+	+	+	+	—
SW ,, ,,	5	10	+	+	+	+	+	+	+	+	+	—
LEE	5	10	+	+	+	+	+	+	+	+	+	—
LEE ,, ,,	5	10	+	+	+	+	+	+	+	+	+	—

* AD—Agglutinating doses.

TABLE II.
Turbidimetric Readings of Virus-Mucin Mixtures.

Materials employed in test: Mucin mg/ml	LEE virus ml	NCAF ml	Saline ml	Photoelectric colorimeter reading Time in hrs at 37°C: 0	1.5	3.25	5	20
2.5	—	—	10	103	110	110	125	101
2.5	5	—	5	103	96	96	107	132
2.5	5	—	5	106	100	96	109	131
2.5	—	5	5	106	95	97	109	108
2.5	—	5	5	95	90	89	97	101
—	5	—	5	42	44	43	43	57

TABLE III.
Effect of Gastric Mucin on Infection of the Chick Embryo with LEE Virus

50% E.I.D.* of virus	Mucin mg/ml	No. eggs	RBC agglutination following inoculation: 48 hrs after	72 hrs after
1	—	6	3/6	4/6
	12.5	6	1/6	2/6
10	—	6	4/6	4/6
	12.5	5	5/5	5/5
100	—	6	6/6	6/6
	12.5	6	2/6	3/6
1000	—	6	6/6	6/6
	12.5	5	5/5	5/5

* E.I.D.—Egg infective dose.

fluenza virus in the developing chick embryo. In Table III are recorded the results obtained when the dilution of virus used in the inoculum is prepared in a solution of mucin containing 12.5 mg/ml of mucin and the mixture held at room temperature for one hour prior to inoculation of 0.05 ml into the allantoic cavity. In the other type of experiment one ml of allantoic fluid was withdrawn from each egg to be inoculated and one ml of the mucin solution injected into the egg one hour prior to inoculation with 0.05 ml of the virus dilution (Table IV). The results recorded in Table III and Table IV show that there is no inhibition of influenza virus multiplication when as much as 25 mg of mucin is introduced into the allantoic cavity prior to inoculation with ten 50% infective doses.

Comment. The evidence presented by Burnet and his colleagues[2,3,4] strongly sug-

[4] Burnet, F. M., *Lancet*, 1948, **1**, 7.

TABLE IV.
Effect of Salivary Mucin on Infection with LEE and PR8 Viruses.

50% E.I.D.* of virus	Mucin mg	Time mucin given in relation to virus inoculation	No. eggs	RBC agglutination 48 hrs after inoculation
100 LEE	5	1 hr before	5	5/5
100 "	—	—	6	6/6
10 PR8	25	1 " "	5	4/5
10 "	—	—	6	6/6

* E.I.D.—Egg infective dose.

gested that influenza virus has a direct enzymic action on mucin in the form of purified blood group substances. Burnet[5] thinks that the chief significance of this finding is that it provides a partial basis for the specific localization of infection by viruses of the mumps-influenza group to glands and mucous surfaces. Our failure to secure any evidence of viral action on human salivary mucin and hog gastric mucin indicates that the chemical composition and/or the molecular configurations of mucins derived from different sources are not identical. Supporting evidence for this viewpoint is contained in a recent report which shows that there are wide variations in the physical characteristics including the isoelectric point of mucin obtained from various levels of the gastro-intestinal tract of the hog.[6] The alteration of the mucinous blood group substances does not result in any alteration of the serological specificity of mucin[2] which again suggests that the change might involve only a slight molecular rearrangement.

Summary. Human salivary mucin and hog gastric mucin do not inhibit the hemagglutination induced by the PR8, LEE, and Swine strains of influenza virus and these mucins do not inhibit the multiplication of PR8 or LEE in the developing chick embryo. There was no turbidimetric or viscosimetric evidence of viral action on these mucins. Influenza virus was also unable to modify the conductivity of the human salivary mucin or the hog gastric mucin.

5 Burnet, F. M., *Austral. J. Science,* 1947, **10**, 21.

6 Domini, G., *Arch. fisiol.*, 1941, **41**, 36.

16349

Effects of Ether and Nembutal Anesthesia upon Blood Concentration of the Rat.

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The effects of ether and nembutal anesthesia on the blood concentration have been reported by several investigators. Anesthesia by nembutal results in hemodilution in cats[1] and in dogs.[2,3] Anesthesia by ether has been found to produce hemoconcentration in dogs[4–7] and

1 Hamlin, H., Essex, H. E., and Mann, F. C., *Am. J. Physiol.*, 1939, **125**, 713.

2 Hausner, E., Essex, H. E., and Mann, F. C., *Am. J. Physiol.*, 1938, **121**, 387.

3 Hahn, P. F., Bale, W. F., and Bonner, J. F., Jr., *Am. J. Physiol.*, 1943, **138**, 415.

4 Barbour, H. G., and Bourns, W., *Am. J. Physiol.*, 1924, **67**, 399.

5 McAllister, F. F., *Am. J. Physiol.*, 1937, **119**, 363; 1938, **124**, 391.

6 Bollman, J. L., Svirbely, J. L., and Mann, F. C., *Surgery*, 1938, **4**, 881.

7 Searls, P. W., *J. A. M. A.*, 1939, **113**, 906.

a similar but less marked effect is believed to appear in man.[8,9] However, in etherized cats[10] and rabbits[11] no such blood concentration occurs.

It is clear that a species difference exists in the response of the above animals to ether, and with further investigation a difference may appear among other experimental animals. In this respect the laboratory rat has been strangely neglected; no study of the effect of ether or nembutal on the blood concentration of the rat has been reported in the literature.

The problem introduced is important on two counts: (1) the correct interpretation of blood data obtained from anesthetized animals requires a knowledge of the changes produced by the anesthesia employed; (2) there is danger in applying information gained from one species to experiments involving another.

Methods. Male albino rats, weighing about 200 g, were studied in 2 groups of 20 rats each, for the effect of anesthesia on the blood concentration. The effects of ether and nembutal were determined on separate groups. Ether was administered by the respiratory route until the animal was prostrate and all reflexes absent. Nembutal was given by intraperitoneal injection in the amount of 0.2 ml of a 10% solution. The rats were placed in a restraining box and blood samples were taken before and immediately after anesthesia by clipping the tail and using the free-flowing blood. Additional samples were obtained following anesthesia by the use of cardiac puncture. Determinations were made on the red cell count and on the specific gravity of the blood. The cell count was determined by employing standard clinical dilution pipettes, diluting fluids, and hemocytometers. The specific gravity was determined by the copper sulfate method of Phillips *et al.*[12]

Results. It is clear from the averages in Tables I and II that both ether and nembutal produced a hemodilution as expressed in the reduced red cell numbers and specific gravity. These differences have been shown to be significant by application of the Fishers' t test. The greater dilution of the blood drawn from the heart as compared to that taken from the tail is due to the longer time over which the anesthesia was allowed to act. This is supported by the fact that in a separate study red cell counts made on blood samples taken simultaneously from the tail and heart of a group of ten anesthetized rats were nearly identical, the means being 7,651,000 and 7,513,000 respectively. Similar counts on a group of ten unanesthetized rats also showed the blood from these two sources to be nearly identical, the numbers being 8,967,000 and 8,790,000.

TABLE I.
Effect of Anesthesia by Nembutal and by Ether on the Erythrocyte Number of the Blood of the Albino Rat. 20 Rats for Each Determination.

Treatment and source of blood	Red blood cells: Mean* × 1000	Red blood cells: Range × 1000
No anesthesia; tail	8,530 ± 130	(7,220-9,780)
Nembutal; tail	7,802 ± 109	(6,430-8,650)
" heart	7,376 ± 136	(6,330-8,180)
No anesthesia; tail	8,368 ± 200	(7,440-9,384)
Ether; tail	7,665 ± 210	(7,106-8,926)
" heart	6,965 ± 88	(6,240-7,910)

TABLE II.
Effect of Anesthesia by Nembutal and by Ether on the Specific Gravity of the Blood of the Albino Rat. 20 Rats for Each Determination.

Treatment and source of blood	Specific gravity: Mean*	Specific gravity: Range
No anesthesia; tail	1.0603 ± .0002	(1.059-1.062)
Nembutal; tail	1.0549 ± .0003	(1.053-1.058)
" heart	1.0529 ± .0003	(1.051-1.055)
No anesthesia; tail	1.0584 ± .0003	(1.058-1.061)
Ether; tail	1.0548 ± .0002	(1.053-1.057)
" heart	1.0521 ± .0001	(1.050-1.055)

* Including standard deviation of the mean.

[8] Gibson, J. G., 2nd, and Branch, C. D., *Surg., Gyn. and Obstet.*, 1937, **65**, 741.

[9] Ragan, C., Ferrebee, J. W., and Fish, C. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 712.

[10] Conley, C. L., *Am. J. Physiol.*, 1941, **132**, 796.

[11] Barbour, H. G., *Anesthesiology*, 1940, **1**, 121.

[12] Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibold, R. M., *Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma*, Josiah Macy, Jr., Foundation, New York, Feb., 1945.

Summary. Anesthesia in the rat by means of nembutal or ether produces a hemodilution, as shown by both a decrease in the red cell numbers and in the specific gravity of the blood.

16350

Possible Eczematous Cross-Hypersentitivity Between Paraphenylenediamine and Azo-Dyes Certified for Use in Foods, Drugs and Cosmetics.*

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In recent reports Dobkevitch and Baer[1,2] have analyzed in a number of patients the cross-sensitization between paraphenylenediamine and certain azo-dyes used in the manufacture of nylon stockings. It was found that this cross-sensitization represented a new example of the complex of hypersensitivity to compounds of quinone structure, as described by Mayer.[3,4] The present study was undertaken to ascertain, in subjects with allergic eczematous contact-type sensitization to paraphenylenediamine, whether this cross-sensitization extends not only to those azo-dyes used in the dyeing of various goods or as therapeutics for dermatological purposes, but also to those azo-dyes which are commonly used in the United States as dyes, for foods, drugs and cosmetics.

The use of synthetic dyes in foods, drugs and cosmetics is regulated under authority of the Federal Food, Drug and Cosmetic Act of 1938 and no coal tar colors other than those listed and described in these regulations may be used in foods, drugs and cosmetics. Among the 116 dyes listed, 18 are accepted for use in foods, 10 of which are azo-dyes. These 10 dyes are "straight" colors, free from all impurities other than a specified maximal allowance (5%) for chlorides and sulfates of sodium; for certain aromatic amines, as o-toluidine, 0.05%, permitted in FD&C Orange No. 1; or xylidine, 0.1%, in FD&C Red No. 32; for certain aromatic hydrocarbons, as betanaphthol, 0.05% in the above-mentioned dyes; and permitting only traces of metals such as arsenic (0.00014%) and lead (0.001%).

All colors used in foods have been examined by the Food and Drug Administration and found to be harmless in the toxicologic sense. The present regulations[5] do not set any standards for ascertaining the eczematogenic potential of dyes to be used in foods, drugs and cosmetics, although all of the certified colors have been tested for sensitizing capacity on the skin of groups of guinea pigs (Calvery[6]).

Experimental. Twenty-five subjects with known allergic eczematous contact-type hypersensitivity to paraphenylenediamine and 21 control subjects with no hypersensitivity to this compound but most of them with hypersensitivity to other non-related substances were given patch tests with the following substances:

* With the technical assistance of Mrs. Dorothy Furman.

† Chief Microbiologist, Ciba Pharmaceutical Products, Inc., Summit, N.J.

1 Dobkevitch, S., and Baer, R. L., *J. Invest. Dermat.*, 1947, **8**, 419.

2 Dobkevitch, S., and Baer, R. L., *J. Invest. Dermat.*, 1947, **9**, 203.

3 Mayer, R. L., *Arch. f. Dermat. u. Syph.*, 1928, **156**, 312.

4 Mayer, R. L., *J. Invest. Dermat.*, 1948, **10**, 389.

5 Federal Security Agency, Food and Drug Administration: Coal-Tar Color Regulations, September, 1940.

6 Calvery, H. O., *Am. J. Pharm.*, 1942, **114**, No. 9.

TABLE I.
Chemical Terminology of Dyes.

No.	Designation	Composition
1	FD&C Red No. 1	Disodium salt of 1-pseudocumylazo-2-naphthol-3,6-disulfonic acid
2	" " " 2	Trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid
3	" " " 4	Disodium salt of 2-(5-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid
4	" " " 32	1-xylylazo-2-naphthol
5	" Yellow No. 3	1-phenylazo-2-naphthylamine
6	" " " 4	1-*o*-tolylazo-2-naphthylamine
7	" " " 5	Trisodium salt of 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-*p*-sulfophenylazopyrazole
8	" " " 6	Disodium salt of 1-*p*-sulfophenylazo-2-naphthol-6-sulfonic acid
9	" Orange No. 1	Monosodium salt of 4-*p*-sulfophenylazo-1-naphthol
10	" " " 2	1-*o*-tolylazo-2-naphthol

(a) Ten azo-dyes certified for use in foods, drugs and cosmetics according to the Federal Food, Drug and Cosmetic Act, (b) paraphenylenediamine, and (c) a sample of nylon stocking material dyed, among others, with azo-dyes.

The procedure adopted for patch testing with these substances was that described by Dobkevitch and Baer.[2] All dyes were used in 2% concentrations in white petrolatum. In many instances the reading of the tests was difficult since the dyes, particularly the red, conferred upon the skin a color sometimes indistinguishable from simple erythema.

The chemical terminology of these dyes is given in Table I.

Results. The results of the patch tests are summarized in Tables II and III.

In the group of subjects who were hypersensitive to paraphenylenediamine (Table II), 4 of the 10 azo-dyes tested elicited strongly positive reactions (2+ to 3+) in several patients: FD&C Red No. 32, FD&C Yellow No. 3, No. 4 and No. 6. Occasional reactions were produced by FD&C Red No. 2 and FD&C Orange Nos. 1 and 2.

In the group of control subjects, not sensitive to paraphenylenediamine, (Table III), 3 of the 10 azo-dyes elicited marked positive (2+) reactions: FD&C Red No. 32, FD&C Yellow No. 3 and FD&C Orange No. 2. Slightly positive reactions ((+) and 1+) were occasionally produced by the dyes FD&C Red Nos. 1 and 4 and FD&C Yellow No. 4.

The 3 dyes which produce the strongest primary reactions in the group of patients not sensitive to paraphenylenediamine are among the 4 dyes which produce the strongest reactions in the group of patients sensitive to paraphenylenediamine. Our inference is that in the concentration used, these dyes are primary irritants.

However, an analysis of the results reveals certain differences between the reactivities of the two groups of patients towards these dyes. Generally, the reactions produced in the non-paraphenylenediamine-hypersensitive control group are not only fewer in number, but they are definitely weaker, 10 1+ to 2+ and 4 2+ reactions being elicited in 210 tests. In the group of the paraphenylenediamine sensitive patients there were 13 1+ to 2+, 5 2+ and 14 stronger positive reactions in 250 tests. Dye FD&C Yellow No. 3, for instance, gave the following results: In the control group, 3 patients had 2+ reactions; all the others showed a weaker reaction or were negative (9 out of 21). In the group of patients sensitive to paraphenylenediamine, 8 patients had 2+ or stronger reactions and only 6 out of 25 were negative. Similar results were obtained with the other dyes.

Contrary to the 3 aforementioned dyes which had produced many positive reactions in both groups, FD&C Yellow No. 6 is not a primary irritant, since it produced one 1+ reaction in only one of the controls but produced 8 reactions, 4 of which were 2+ to 3+, among the patients sensitive to paraphenylenediamine.

Discussion. The tests reported in Table III on patients not sensitive to paraphenylenediamine show that the dyes FD&C Yellow No. 3, Orange No. 2, Red No. 32 and to a lesser extent, Yellow No. 3 must be considered as primary irritants in the concentra-

TABLE II.
Patch Tests in Subjects Hypersensitive to Paraphenylenediamine.

Patient	FD&C Red No. 1	FD&C Red No. 2	FD&C Red No. 4	FD&C Red No. 32	FD&C Yel. No. 3	FD&C Yel. No. 4	FD&C Yel. No. 5	FD&C Yel. No. 6	FD&C Or. No. 1	FD&C Or. No. 2	Para-phenylene-diamine	Nylon Stock-ings
F21768	0	0	0	+	+++	(+)	0	++-+++	0	+	++++	+++
F26922	0	0	0	(+)	+-++	(+)	0	+-++	0	++-+++	++++	+++++
C17908	0	0	(+)	+	++-+++	+	0	++-+++	0	+-++	++++	+++
F23260	?	?	?	+-++	++-+++	?	0	++-+++	0	(+)	++++	+++
F27324	0	0	0	+-++	0	0	0	0	0	0	+-++	+++
E91914	0	0	0	+	+-++	0	0	0	0	0	++++	+++
F34347	0	+	0	+	+	0	0	+-++	(+)	(+)	+++	++
F33237	0	0	0	0	0	++	++	+	0	0	+++	++
F37037	0	0	0	+	+++	++-+++	0	+	0	+-++	++++	++
F32987	0	0	0	0	+	0	0	0	0	+	+	++
F29973	0	0	0	+	+	0	0	0	0	+-++	+-++	++
F33388	0	0	0	0	0	0	0	0	0	+	++	++
E51513	0	0	0	+++	+++	0	0	0	0	++	++-+++	++
F39596	0	0	0	+	(+)	0	0	0	+	+-++	+++	++
F35345	0	0	0	+	0	0	0	0	0	0	+++	+-++
F29970	0	0	0	+	(+)	0	0	0	0	+	++	+
F26266	0	0	0	0	++	0	0	0	0	0	+++	—
F27324	0	0	0	(+)	+	+	0	0	0	+	+-++	—
D10124	0	0	0	(+)	+	?	0	0	0	+-++	+++	—
F44406	0	0	0	+	(+)	?	?	++-+++	0	?	++-+++	0
D22965	0	0	0	?	0	(+)	0	0	0	(+)	++-+++	0
F44385	0	0	0	?	(+)	(+)	0	0	0	+	++	0
F44384	0	?	?	++-+++	++-+++	+	?	?	?	+-++	++	?
F44400	0	0	?	?	0	0	0	0	0	0	++-+++	?
F44932	0	0	0	+	++	0	0	0	0	+-++	++	0

TABLE III.
Patch Tests in Subjects *Not* Hypersensitive to Paraphenylenediamine (Control Group).

Patient	FD&C Red No. 1	FD&C Red No. 2	FD&C Red No. 4	FD&C Red No. 32	FD&C Yel. No. 3	FD&C Yel. No. 4	FD&C Yel. No. 5	FD&C Yel. No. 6	FD&C Or. No. 1	FD&C Or. No. 2	Para-phenylene-diamine	Nylon Stock-ings
F31491	0	0	0	0	0	0	0	0	0	0	0	0
F31702	0	0	0	0	0	0	0	0	0	+	0	0
F19511	(+)	0	+	+	++	(+)	0	+	0	+-++	?	0
F40196	+	0	0	0	0	0	0	0	0	0	0	0
C46823	0	0	0	0	+	(+)	0	0	0	+	0	0
F40521	(+)	0	0	(+)	0	+	0	0	0	+	0	0
F41148	0	0	0	+	+	(+)	0	0	0	+	0	0
F41871	0	0	0	+	+	?	0	0	0	+	0	0
F43607	0	0	0	0	0	0	0	0	(+)	0	0	0
A54758	0	0	0	0	?	0	0	0	0	?	0	0
F44451	0	0	(+)	+	+-++	+	0	0	0	+	0	0
F44021	0	0	0	(+)	+-++	0	0	0	0	+	0	0
F44304	0	0	0	?	0	0	0	0	0	(+)	0	0
F45131	?	0	0	+-++	0	0	0	0	0	+	(+)	0
F44868	0	0	(+)	(+)	++	0	0	0	0	?	0	0
F15719	0	0	+	+	+	0	0	0	0	+-++	0	0
F44462	0	0	0	+	+	0	0	0	0	+	0	0
F38568	0	0	0	+-++	++	+	0	0	0	++	0	0
F44014	0	0	0	?	?	0	0	0	0	+	0	0
F45131	0	0	0	+	+	0	0	0	0	+-++	0	0
F45521	0	0	0	+-++	+-++	?	0	0	0	+-++	0	0

tion chosen for the tests. One could therefore question whether positive skin reactions elicited with these dyes in the group of patients sensitive to paraphenylenediamine are expressions of an allergic cross-sensitivity to compounds of quinone structure and not merely coincidental reactivities produced by primary irritants.

Two facts, however, favor the supposition that these reactions are etiologically associated with the sensitivity to paraphenylenediamine and not merely coincidental. (1) Both the number and the strength of the skin reactions produced by these dyes are definitely greater in the group of patients sensitive to paraphenylenediamine than in the control group consisting of patients not sensitive to paraphenylenediamine, but sensitive to other allergens. These dyes elicited 14 reactions stronger than 2+ in the patients hypersensitive to paraphenylenediamine; and no reactions stronger than 2+ in the control group. (2) Although dye FD&C Yellow No. 6 was not a primary irritant in the concentration employed, it produced skin reactions in 8 of the patients sensitive to paraphenylenediamine. In 5 of these cases the strength of the reactions closely paralleled their reactions to paraphenylenediamine. We therefore believe that among the positive reactions produced by the various dyes in paraphenylenediamine sensitive patients, there are several elicited by a specific skin sensitivity to compounds of quinone structure.

The results obtained suggest that the eczematous hypersensitivity to paraphenylenediamine not only crosses over to some azo-dyes which are used for dyeing leather, fabrics and other goods, but also to certain food dyes, such as FD&C Yellow No. 6 which is commonly used for coloring beverages, FD&C Red No. 32 used in external coloring of oranges and FD&C Yellow Nos. 3 and 4, both used in oleomargarine and butter.

It is by no means surprising that certain azo-dyes employed in coloring food are capable of producing cross-reactions in patients sensitive to paraphenylenediamine. From the chemical standpoint these food colors are not fundamentally different from those azo-dyes which are used for technical or pharmaceutical purposes, their only distinctive property being their specific purpose and the fact that they are more highly purified.

For obvious reasons these azo-dyes are less conspicuous as general allergens than the related azo-compounds which are used for other purposes. The production of sensitizations and the elicitation of the reactions of hypersensitivity depend to a great extent not only on the intrinsic sensitizing power of the antigen, but also on the concentration and on the intensity with which the material comes in contact with the susceptible cells.

There are indeed quantitative differences in the amounts of dye usually absorbed by the body from foods and those coming in contact directly with the skin from dyed goods. The quantities of azo-dyes ingested with each meal and then reach the cells of the skin must be much smaller than those which reach the skin from dyed fabrics, furs or from medicinal preparations.

As is often the case under similar circumstances, the question arises whether the positive patch tests elicited by the food dyes in patients sensitive to paraphenylenediamine are of practical importance. It remains to be shown that patients presenting these positive reactions will react with clinical symptoms to the ingestion of foods colored with the incriminated dyes.

We have had one patient, sensitive to paraphenylenediamine, eat within 48 hours, one pound of oleomargarine dyed with dye FD&C Yellow No. 3 but the ingestion was not followed by any clinical manifestations of hypersensitivity. However, further trials of this type and particularly chronic and repeated exposures by ingestion are necessary; only then will it be known whether the amount of azo-dye ingested with food is sufficient to elicit reactions in individuals sensitized to compounds of quinone structure or will cause sensitizations in normal individuals. This problem is worthy of serious consideration since a very large portion of the population of the United States is continually exposed to many of the certified azo-dyes by contact, ingestion and probably also inhalation and

the possibility of their reacting to these substances may constitute a serious problem.

Certain other problems are intimately related to this question. It is known, for example, that a number of cases of dermatitis from paraphenylenediamine and related compounds used in hair and fur dyes, photo developers, leather dyes, nylon stockings, etc. occasionally show flare-ups which cannot be explained on the basis of a new exposure to the causative agent. Our results suggest the possibility that the ingestion of certified azo-dyes, which have been shown to produce reactions in patients hypersensitive to paraphenylenediamine, may be responsible for these recurrences. Furthermore, it may be possible that certain eczematous eruptions now attributed to various foods themselves are actually due to the azo-dyes contained in these foods. A study of the influence of continuous exposure to these dyes on the causation and persistence of non-eczematous and possibly non-cutaneous allergies as, for example, asthma due to paraphenylenediamine and related compounds, would prove of great interest.

In evaluating the results shown in Table II it seems at first glance inexplicable that not all aminated azo-dyes used in foods, cosmetics and drugs produce irritations. While certain of the food dyes listed in Table II are seemingly harmless, even for patients highly sensitive to paraphenylenediamine, other dyes of closely related constitution elicit strong skin reactions in a high percentage of cases. The data gathered to date in various studies indicate that the presence or absence of a response to a given azo-dye in a case of cross-sensitization to compounds of quinone structure depends essentially upon the chemical structure of the dyes. Indeed, the capacity of an azo-dye to produce skin reactions in patients sensitive to paraphenylenediamine seems to be dependent in most cases upon the ease of its transformation into compounds of quinone structure and upon the ability of the quinone compound thus formed to couple with certain body constituents. We believe that only those azo-dyes capable of undergoing these changes have sensitizing power and elicit reactions in an appreciable number of cases.[4]

Conclusions. Skin tests with certain certified food dyes in patients sensitive to paraphenylenediamine suggest that eczematous hypersensitivity to paraphenylenediamine may not only cross over to azo-dyes used for the dyeing of leather and fabrics, or in ointments, but also to certain azo-dyes certified by the Food, Drug and Cosmetic Act of 1938 for use in foods, drugs and cosmetics. The implications of these findings are discussed.

16351

Effect of Pneumonectomy and of Lung Extract on Experimental Renal Hypertension.*

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Katz and Steinitz[1] have demonstrated in dogs that pulmonary arterial pressure is not altered in experimental renal hypertension. This may be explained by the inability of the pulmonary arterioles to respond adequately to the renal pressor mechanism, inasmuch as Brenner[2] found that the walls of these vessels average 5.7% of the external

* Aided by grants from the John and Mary R. Markle Foundation, the United States Navy, and the Graduate School Research Board of the University of Illinois.

1 Katz, L. M., and Steinitz, F. S., *Am. J. Physiol.*, 1940, **128**, 433.

2 Brenner, O., *Arch. Int. Med.*, 1935, **56**, 211, 457, 724, 976, 1189.

diameter, compared with 36% for the systemic arterioles. Certain experimental data, however, demonstrate that the pulmonary arterioles do respond to pressor agents. Friedberg *et al.*[3] showed that the intravenous injection of angiotonin produced a rise in pressure in the pulmonary artery of the dog occurring almost simultaneously with the systemic rise. With renin, the pulmonary pressure rise lagged behind the systemic. Binet and Bergeton[4] observed a vasoconstrictor action of renin on the vessels of the isolated dog lung, and found that renin is inactivated by passage through a heart-lung preparation. Braun-Menendez *et al.*,[5] however, observed no appreciable diminution in renin activity after 2 hours of perfusion through a heart-lung preparation. These findings suggest either that renin and angiotonin are not involved in hypertension of renal origin, or that in chronic renal hypertension the pressor mechanism may operate differently in the pulmonary as compared with the systemic circulation.

Design of experiments. The pulmonary normotension in experimental renal hypertension may be due to the elaboration by the lung of an antihypertensive substance or to the lung's inactivation of a pressor agent produced by the kidney. This may occur to an extent sufficient to prevent the development of pulmonary hypertension, yet not to inhibit the production of an elevated pressure in the systemic circuit.

To test for an antihypertensive agent in lung tissue, 2 dogs were injected with lung extract for 2 months, then were subjected to a unilateral renal artery constriction and a contralateral nephrectomy. Injections were continued throughout the operation period and for 2 months following. Good evidence exists that prophylaxis can be obtained by a similar injection procedure with certain renal extracts.[6] The technique used in constricting the renal arteries has been found to result in the development of hypertension in a high percentage of control dogs.

To investigate the effect of a reduction in lung substance, pneumonectomy was performed on 4 dogs with moderate renal hypertension. Two of these dogs were later subjected to contralateral lobectomy. It was felt that any possible pressor inactivating effect of the lungs might thereby be reduced to a degree sufficient to permit the hypertensive substance elaborated by the kidney to raise the systemic pressure to a still higher level. Two normotensive dogs were pneumonectomized as controls. Unilateral renal artery constriction and contralateral nephrectomy were later done on these controls.

Procedures. Arterial pressures were obtained on the animals by direct femoral puncture at least twice a week.[7] The renal arteries were clamped according to our modification of the Goldblatt technique[8] which produces hypertension in practically 100% of dogs. The pneumonectomies and lobectomies were performed according to the technique of Joannides.[9]

The lung extract was prepared from dog lungs removed immediately after death. The lungs were frozen and ground in a meat chopper. The tissue was then defatted over 48 hours by 2 changes of cold acetone and for another 24 hours by cold ether. It was then dried under vacuum at room temperature in a drying column and finely divided in a coffee grinder. Fresh extracts of this lung powder were made with a .5% $NaHCO_3$ in physiological salt solution. One cc of the final extract was equivalent to one gram of fresh tissue. Streptomycin and penicillin were added as preservative. The animals were injected intramuscularly 6 days a week with a dose of 1.5 cc per kg of body weight.

3 Friedberg, L., Katz, L. N., and Steinitz, F. S., *J. Pharmacol. and Exp. Therap.*, 1943, **77**, 80.

4 Binet, L., and Bergeton, D., *C. R. Soc. de Biol.*, 1942, **136**, 134.

5 Braun-Menendez *et al.*, *Renal Hypertension* (translated by L. Dexter), C. C. Thomas, Springfield, Ill., 1946, p. 207.

6 Wakerlin, G. E., Johnson, C. A., Smith, E. L., Moss, W. G., and Weir, J. R., *Am. J. Physiol.*, 1942, **137**, 515.

7 Dameshek, W., and Loman, J., *Am. J. Physiol.*, 1932, **101**, 140.

8 Wakerlin, G. E., unpublished data.

9 Joannides, Minas, *Arch. Surg.*, 1928, **17**, 91.

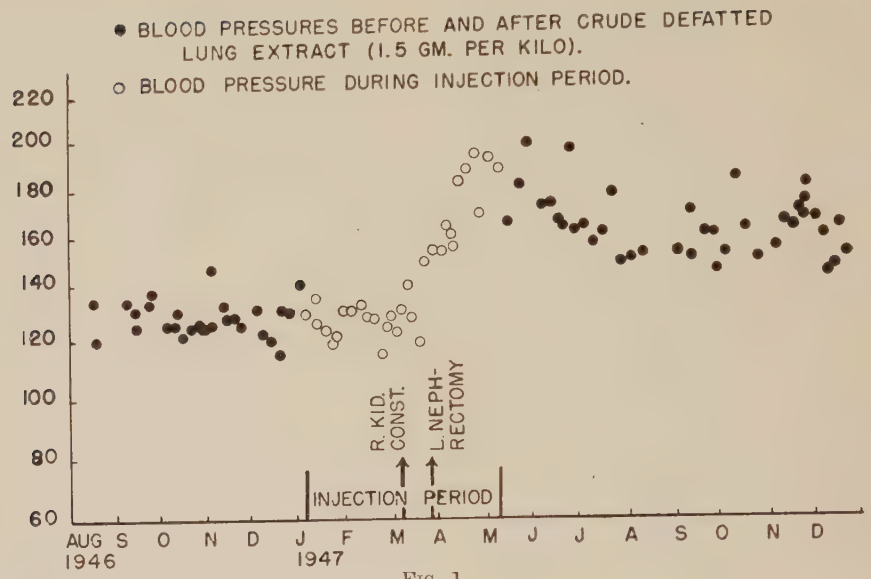

FIG. 1.

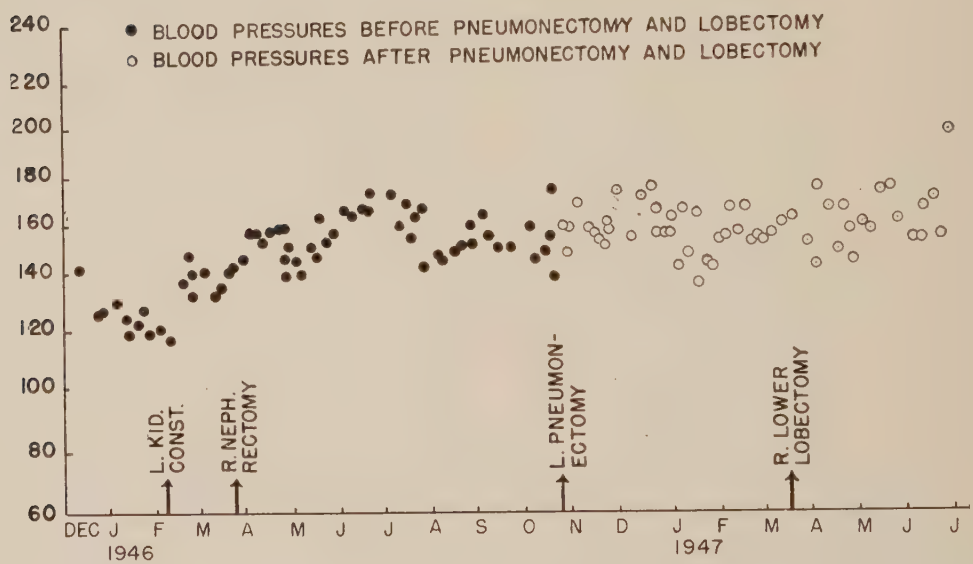

FIG. 2.

Results. 1. The lung extract failed to prevent the development of hypertension in the 2 injected dogs following constriction of the renal artery and contralateral nephrectomy (Fig. 1). All ten of the control dogs developed hypertension, 2 of them dying of malignant hypertension. 2. Pneumonectomies performed on 2 of the 4 moderately hypertensive dogs did not alter in any way their pressure over a period of 4 months. 3. Pneumonectomy and contralateral lobectomy on the other 2 hypertensive dogs likewise did not alter their pressure over a period of 6 months (Fig. 2). 4. The normotensions of 2 dogs were not influenced by pneumonectomy, nor did the absence of a lung alter the hypertension produced by subsequent renal artery clamping and contralateral nephrectomy.

Discussion. The results demonstrate that the lung extract used had no prophylactic

antihypertensive effect. Moreover, a preliminary experiment with the dog lung extract in rats likewise indicated no prophylaxis against experimental renal hypertension.[10] However, antihypertensive substances not obtained by this method of extraction may be present in lung.

A reduction in the amount of functioning lung tissue was shown to be without effect on the systemic blood pressure of moderately hypertensive animals. No regeneration of lung tissue was found in these dogs on sacrifice, and it seems likely that sufficient tissue was removed to demonstrate some effect if the lung plays an active antipressor role in experimental renal hypertension.

Summary. 1. Daily intramuscular injections of lung extract for two months preceding and following clamping of the renal artery and contralateral nephrectomy failed to prevent the development of hypertension in two dogs. 2. Pneumonectomy performed on 2 renal hypertensive dogs and pneumonectomy plus contralateral lobectomy on 2 other hypertensive animals did not alter their pressure levels. 3. The pressures of 2 normotensive dogs were not influenced by pneumonectomy, nor were the hypertensions subsequently produced by renal artery constriction and contralateral nephrectomy. 4. The results of these experiments suggest, but do not prove, that the lungs do not elaborate an antihypertensive substance and do not inactivate the pressor agent of experimental renal hypertension. 5. Further work is necessary to explain the pulmonary normotension of renal hypertensive dogs.

We are grateful to Dr. M. Joannides for performing the pneumonectomies and lobectomies.

[10] Klatch, B. Z., unpublished data.

16352

Relationship Between the pH of the Duodenal Content and Pancreatic Secretion.*

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Bayliss and Starling[1] demonstrated the mechanism whereby acid introduced into the intestine provoked a flow of pancreatic secretion. With increasing interest in the role of the endocrine glands in the regulation of bodily function it has been widely assumed that the secretin mechanism largely determined the volume of pancreatic secretion.[2] Farrell and Ivy,[3] who feel the secretin mechanism is the most important, found that a denervated pancreas failed to secrete as much as expected. McClure,[4] on the basis of his determination of enzyme in the duodenal content, stated that "stimulation of the external enzymic function of the pancreas is dependent on some factor or factors unrelated to acidity of intestinal contents." Thomas

* The work was supported in part by a grant from the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions and conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

[1] Bayliss, W. M., and Starling, E. H., *Am. J. Physiol.*, 1902, **28**, 325.

[2] *Howell's Text Book of Physiology*, 15th Edition, 1946, p. 1064, W. B. Saunders Co., Philadelphia; *Physiology in Health and Disease*, C. S. Wiggers, 4th Edition, 1947, p. 809, Lea and Febiger, Philadelphia.

[3] Farrell and Ivy, *Am. J. Physiol.*, 1926, **78**, 325.

[4] McClure, C. W., *Functional Activities of the Pancreas and Liver*, Medical Authors Publishing Co., 1937.

[5] Thomas, J. E., and Crider, J. O., *Am. J. Physiol.*, 1940, **131**, 349.

and Crider[5] found that acid, as a stimulant for pancreatic secretion was effective when the pH was about 4; however, a copious flow of pancreatic juice was produced only when the pH was reduced to 3 or less. The latter investigators also[6] found that, in the dog, after a meat meal, the pH of the duodenal content generally hovered around 4, at which level, a threshold response only would be expected.

Since pancreatic juice with a pH of about 8 is secreted into a vulnerable part of the duodenum, and is an excellent buffer, it is generally believed that this secretion plays a decisive role in the neutralization of acid chyme.[7] Hoerner[8] showed that even when the pancreatic juice was excluded from the duodenum, a significant degree of neutralization of the acid gastric contents occurred. On the other hand, DeBakey[9] felt that the pancreatic juice played a lesser role than did bile in the prevention of ulcer in the intestinal tract.

In the course of study undertaken to determine the normal secretion of the pancreas after the ingestion of various foodstuffs, observations were made which yielded information pertaining to both of these problems.

Methods. Healthy adult dogs were provided with gastric and duodenal tubulated fistulae as has been described elsewhere.[10] The accessory pancreatic duct was doubly ligated and severed; the duodenal fistula was placed opposite the ostium of the main pancreatic duct. (Fig. 1). After complete recovery from the above operation, the animal was placed in a standing position on a table by means of a comfortable muslin harness. A glass cannula was introduced into the main pancreatic duct; two tubes were placed into the stomach and then through the pylorus into the duodenum, the shorter tube being so placed that its end was in the region of the orifice of the main pancreatic duct while the end of the longer tube was placed 3 inches beyond this point. The gastric fistula was stoppered and the duodenal fistula was covered with a rubber dam which was pierced to permit the small pancreatic cannula to protrude to the outside.

Observations were made on 3 dogs so prepared. The pancreatic juice was collected and the volume measured every 10 minutes. In one series of experiments, each 10-minute specimen was introduced into the duodenum through the proximal tube; in a second group the pancreatic juice was excluded from the intestine during the period of observation. Samples of duodenal content were withdrawn from the longer tube at intervals and the pH determined electrometrically, using a glass electrode. These samples were then promptly returned to the duodenum. Preliminary observations were made in the fasting state for one hour, after which the animal was fed small amounts of different foodstuffs and observations were continued for five hours. In a few experiments large meals were fed, and observations continued for a longer period of time.

Results. A comparison of the pH determinations of the duodenal contents made when the pancreatic juice was excluded from the intestine with the observations made when it was reintroduced, reveals that although, in general, the pH is lower when the juice is not present, the acidity did not exceed that observed when the juice was replaced into the duodenum. The difference between the 2 groups of experiments is evident when individual foodstuffs are considered separately. There is a tendency for the pH to be higher when the pancreatic juice is present.

Fig. 2 presents this data *in toto*. The ordinates represent the pH determined after the ingestion of food, the abscissae denote the volume of pancreatic juice secreted in the ensuing 10-minute period. Entirely similar results were obtained when the volume of pancreatic juice secreted during the same ten-

6 Thomas, J. E., *Am. J. Dig. Dis.*, 1940, **7**, 195.

7 Mann, F. C., and Bollman, J. L., *Am. J. Dig. Dis.*, 1935, **2**, 284; Hoerner, M. T., *Am. J. Dig. Dis.*, 1935, **2**, 300.

8 Hoerner, M. T., *Am. J. Dig. Dis.*, 1935, **2**, 288.

9 DeBakey, M. E., *Arch. Surg.*, 1937, **34**, 230.

10 Scott, V. Brown, Collingnon, W. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 248; Hart, Wm. M., and Thomas, J. E., *Gastro-enterology*, 1945, **4**, 409.

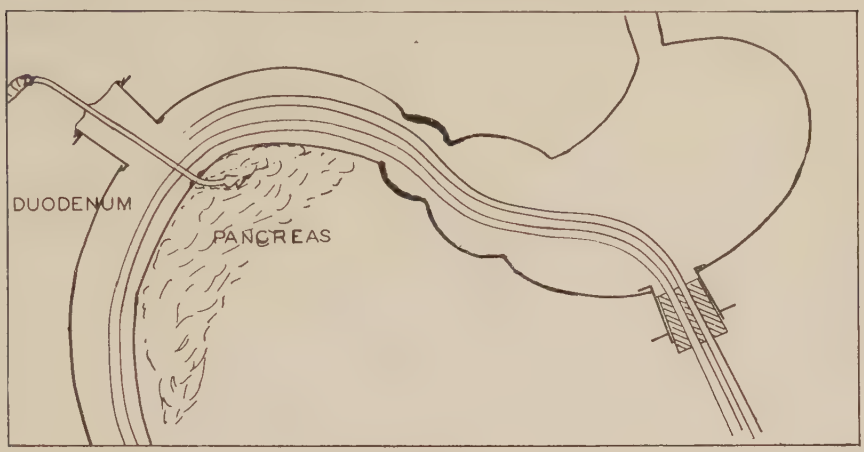

FIG. 1.

Diagram showing relationships of the gastric and duodenal fistulas and the pancreatic cannula; also, the position of the two tubes introduced into the duodenum.

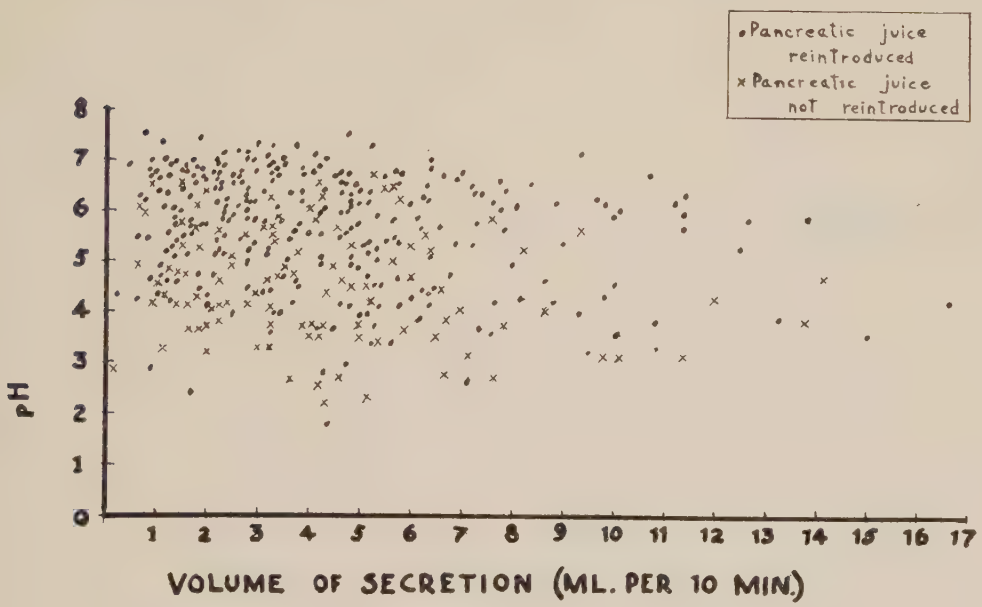

FIG. 2.

Scattergram showing the relationship between the pH of the duodenal content and the volume of pancreatic juice secreted during the ensuing 10-minute period.

minute period was plotted against the pH. Dots represent determinations made when the pancreatic juice was returned to the duodenum, crosses represent similar determinations made when the juice was withheld for the entire period of observation. The range of the pH was lower, but did not exceed the limits observed when the pancreatic juice was present in the duodenum. It is evident that the pancreatic juice exerts only a moderate effect on the pH of the intestinal content.

The same data may be used to study the effect of acid in the intestine on the volume of pancreatic secretions. It is evident from a glance at this graph that no clear correlations between the volume of pancreatic secretion and the pH of the duodenal content existed in these experiments. This is rather strikingly illustrated by individual experi-

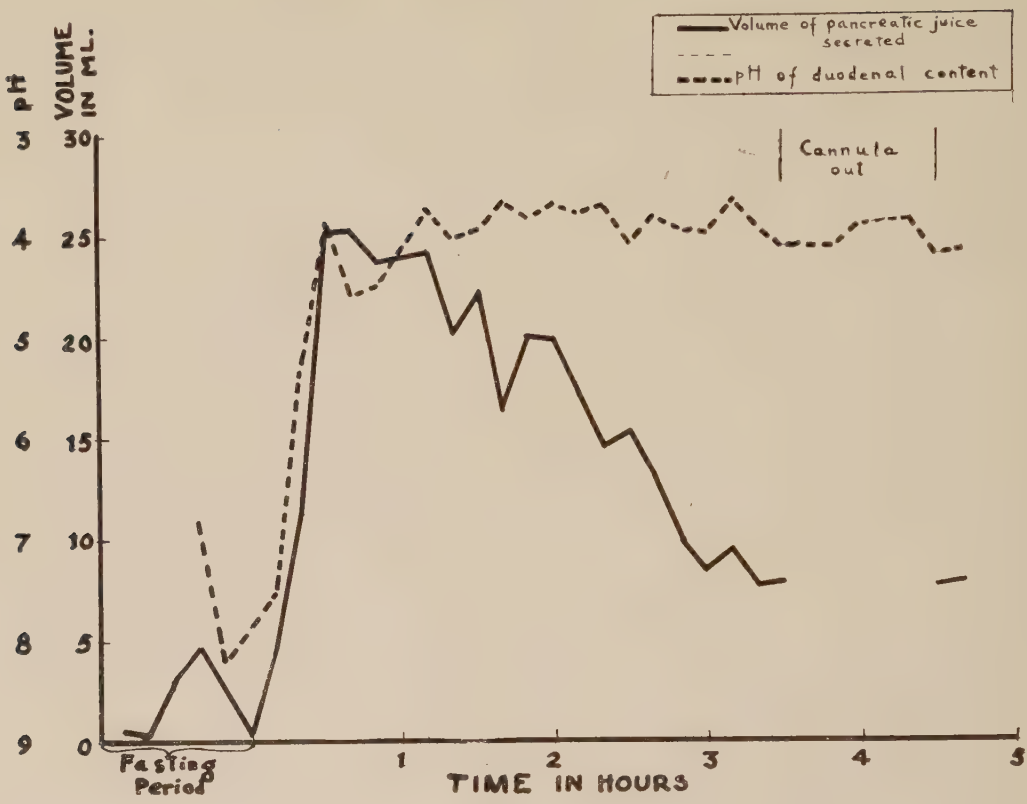

FIG. 3.

Curves of a typical experiment after the feeding of a large meal of meat, showing the relatively constant pH of the duodenal content in relation to the marked variation in the secretion of pancreatic juice at 10-minute intervals.

ments. Fig. 3 illustrates the results obtained when a large meal of meat was fed. It will be noted the pH of the duodenal content throughout the period of observation hovered about 4, whereas, the volume of pancreatic juice varied considerably. Fig. 4 represents data obtained after a small feeding of bread. It does not seem likely that the fluctuations in the volume of the pancreatic juice were related to the differences in pH observed.

Comment. Our observations are in conformity with those of Hoerner; namely, that some neutralization of the acid chyme is attained by the action of bile, succus entericus and other secretions of the small intestine and its appendages, but that generally more complete neutralization results when pancreatic juice is present in the intestine.

In addition, our findings would suggest that during digestion, the volume of pancreatic juice secreted is determined by other factors than the acidity of the duodenal content alone. It would seem plausible that the secretin mechanism may "prime the pancreatic pump," since the duodenal acidity is at, or may exceed, the threshold value. However, other factors must play a determining role. Thus Crider and Thomas[11] found that, after section of the vagus nerves, the introduction of acid into the intestine was less effective in promoting pancreatic secretion than previously. Possibly two or more different stimuli must be active at any one time to evoke a copious pancreatic secretion, as does occur during the digestive period. However, it would seem that when the duodenal acidity exceeds a given threshold value, as does oc-

[11] Crider and Thomas, *Am. J. Physiol.*, 1944, **141**, 730.

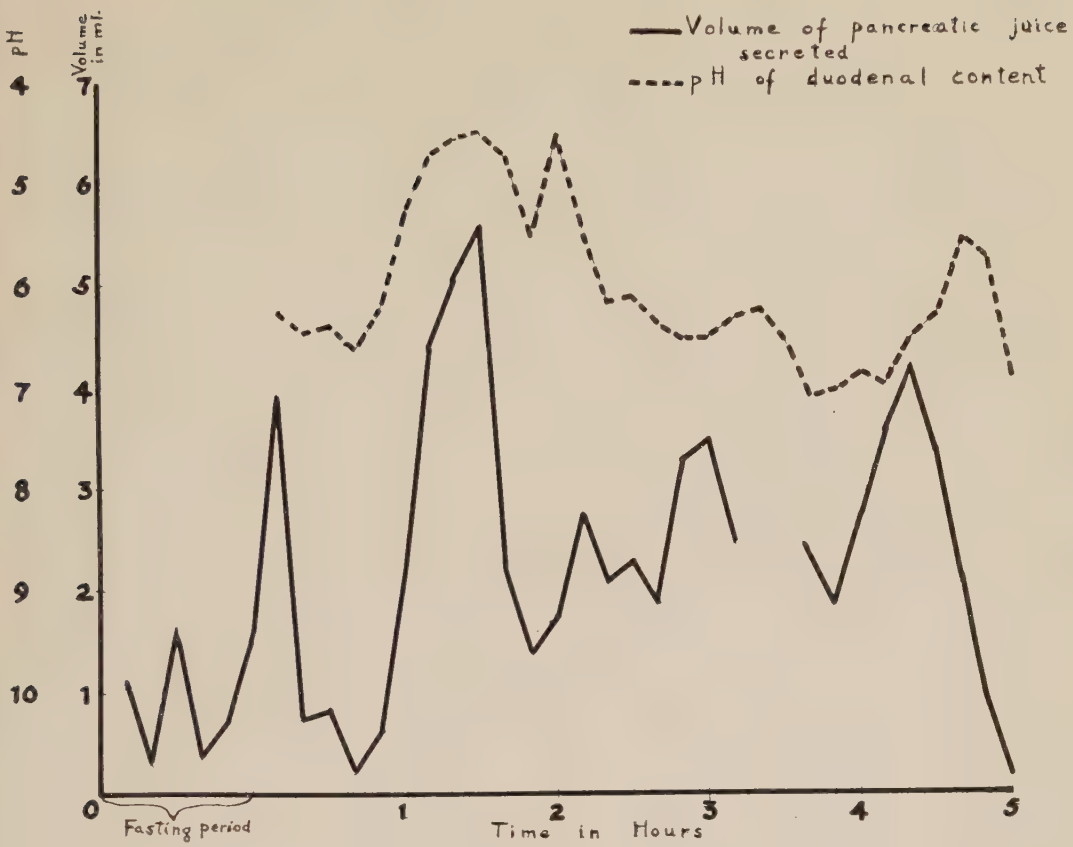

FIG. 4.

Curve showing a typical experiment after small feeding of bread, demonstrating the lack of relationship between the pH of the duodenal content and the secretion of pancreatic juice at any given time. Determinations were made at 10-minute intervals.

casionally occur, the secretin mechanism may call forth a larger volume of neutralizing pancreatic juice.

Conclusions. 1. Pancreatic juice provides buffering material which serves to neutralize gastric content more effectively, but some degree of neutralization is attained from other secretions of the small intestine and its appendages.

2. No correlation is evident between the acidity of the duodenal content and the volume of pancreatic secretion.

16353

In vitro Lysis of Leucocytes from Tuberculous Humans by Tuberculoprotein.*

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In a study on the delayed type of hypersensitivity, it has been found that lymphocytes from tuberculous mice and guinea pigs are specifically destroyed when exposed *in vitro* to tuberculoprotein.[1] The purpose of this report is to extend this type of observation to man.

Experimental. Using a method previously described,[2] suspensions of human white cells were obtained by layering whole, heparinized blood over albumin-saline mixtures,‡ and centrifuging. The resulting cell suspensions consisted of 50-80% lymphocytes, 20-40% neutrophil leucocytes, and small percentages of the other white blood cells. Experiments were performed on the leucocytes from 3 groups of subjects: (1) tuberculin-positive (PPD) patients with bacteriologically proven, active, severe tuberculosis; (2) subjects with positive tuberculin skin tests (PPD) but no demonstrable clinical tuberculosis; and (3) healthy persons with negative tuberculin skin tests (PPD, second strength).

Cytolysis was demonstrated by adding 0.2 cc of white cell suspension (7,000-15,000 cells per cu mm in fresh normal human serum) to 0.2 cc of "antigen" solution (fresh normal human serum containing PPD-s,[3] 25 γ per cc). Directly after mixing, these preparations were incubated in a water-bath at 37°C. Samples were taken at 5, 20, 60 (and 90) minutes for total white cell counts (5-10% error), and differential counts (1000 cells).

As cell system controls, parallel counts were performed on similar cell concentrations suspended in fresh normal human serum alone. Cell preparations suspended in fresh normal serum containing 200 γ per cc of a beta streptococcal protein, similar to that used to demonstrate cytolysis in tissue culture,[4] served as "antigen" controls.

It was previously noted that phosphate or citrate ions blocked cytolysis in this type of experiment.[1] To further elucidate the role of complement in this reaction, fresh human serum heated at 56°C for one-half hour, was used in some experiments as the suspending and diluting fluid, in place of fresh normal human serum.

Experiments have been performed one or more times on at least 6 individuals in each of the 3 categories outlined.

Results. Sample protocols illustrate the results obtained in typical experiments.§

1. Twenty to 60% of the lymphocytes, and 20-90% of the neutrophil leucocytes from the peripheral blood of patients with active severe tuberculosis, are specifically destroyed within 90 minutes by contact *in vitro* with small amounts of tuberculoprotein (Table I).

2. If heat-inactivated serum is substituted for fresh normal serum as the suspending and diluting fluid in these preparations, no cytolysis occurs (Table I).

3. No cytolysis, beyond that induced by the trauma of the procedure (1-10% in 2 hours), occurs on exposure of these cells to

* Work done under an U.S.P.H.S. Research Grant.

† Francis Weld Peabody Fellow in Medicine.

‡ Sterile 35% isotonic bovine albumin solution kindly supplied by Armour and Company, Stock Yards, Chicago, Ill.

1 Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 269.

2 Ferrebee, J. W., and Geiman, Q. M., *J. Inf. Dis.*, 1946, **78**, 173, as modified by Favour.[1]

3 Seibert, F. B., and Glenn, J. T., *Am. Rev. Tub.*, 1941, **44**, 9.

4 Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

§ In the protocols, eosinophils, basophils, and monocytes have not been recorded as they total less than 2%.

TABLE I.
In vitro Effect of PPD on Blood Leucocytes from a Patient with Miliary Tuberculosis (Tuberculin Positive).

			Lymphocytes		Granulocytes	
		Total WBC	%	Absolute No.	%	Absolute No.
PPD	5′	2430	77.1	1880	21.8	530
Normal serum	30′	1260	88.1	1080	11.0	136
	60′	810	87.5	700	11.4	92
PPD	5′	3190	66.2	2110	33.6	1070
Heated serum	30′	3270	65.2	2130	34.8	1140
	60′	3280	70.0	2290	30.0	990
Normal serum	5′	2580	78.4	2020	21.3	550
control	30′	2380	79.0	1880	20.1	480
	60′	2570	79.7	2050	19.6	500

Note: Progressive cytolysis of lymphocytes and granulocytes in the presence of PPD and fresh normal human serum. No cytolysis with heat-inactivated serum.

TABLE II.
In vitro Effect of PPD on Blood Leucocytes from a Patient with Tuberculous Pneumonia (Tuberculin Positive).

			Lymphocytes		Granulocytes	
		Total WBC	%	Absolute No.	%	Absolute No.
PPD	5′	8890	53.2	4730	46.6	4140
Normal serum	20′	8130	52.0	4220	47.7	3880
	60′	7220	56.6	4080	43.3	3120
	90′	6160	52.4	3220	46.9	2890
Strep. protein	5′	8130	57.4	4670	42.2	3430
Normal serum	20′	8440	56.0	4730	44.0	3710
	60′	8060	60.0	4840	40.0	3220
	90′	8120	56.4	4580	43.2	3500
Normal serum	5′	7790	58.0	4520	41.6	3240
control	20′	7450	59.0	4400	41.0	3050
	60′	7030	60.0	4220	40.0	2810
	90′	7320	58.2	4260	41.6	3050

Note: Progressive cytolysis of lymphocytes and granulocytes in the presence of PPD and fresh normal human serum. No cytolysis with strep. protein (200 γ per cc).

(a) fresh normal human serum alone, or (b) streptococcal protein in fresh normal serum (Table II).

4. Leucocytes from healthy tuberculin-positive or tuberculin-negative subjects are not affected by contact *in vitro* with PPD or streptococcal protein, under the conditions of these experiments.

Conclusions. The observation that lymphocytes from animals infected with certain bacteria are specifically destroyed by contact *in vitro* with protein extracts of these bacteria[1] has been extended to humans infected with *M. tuberculosis*. In the human, as in the guinea pig, the neutrophil leucocytes are also specifically lysed. It is of interest that although the lymphocytes from tuberculous subjects of all 3 species (mouse, guinea pig, human) so far studied, are specifically sensitive to tuberculoprotein, the neutrophil leucocytes are affected only in those species which show a positive delayed-type tuberculin skin test (*i.e.*, guinea pigs and humans). In the tuberculous mouse, which fails to develop delayed-type skin hypersensitivity reactions to tuberculoprotein,[5] lympholysis occurs but

[5] Gerstl and Thomas, *Yale J. Biol. and Med.*, 1940-41, **13**, 679, ref. cit.

other cell types remain unaffected.[1] Apparently, the presence of complement is necessary for the occurrence of this form of prompt cytolysis. Experiments to further elucidate the role that this type of cytolysis plays in the pathogenesis of delayed-type hypersensitivity are in progress.

16354 P

An Electrophoretic Study of the Serum Proteins in Scleroderma.

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Scleroderma (literally: "hardening of the skin") occurs in two forms: diffuse and localized. The diffuse form is a progressive systemic disease of the connective tissue throughout the body.[1,2] The localized form on the other hand appears clinically to be limited to the skin and is a much milder disease. In spite of numerous investigations, the pathogenesis of scleroderma remains obscure. Observations on the serum proteins have been limited to an occasional routine determination showing a decrease in the albumin/globulin ratio with little change in the value for total proteins.[3] It seemed possible that a more detailed analysis by the Tiselius method of electrophoresis might yield additional information of value.

Methods and Materials. Serum was obtained in the fasting state from 12 normal subjects, 5 patients with diffuse scleroderma, and one patient with localized scleroderma. Four ml of serum were dialyzed against 2 liters of barbiturate buffer (ionic strength 0.1, pH 8.6) for 48 hours at 4°C, then diluted 1:3 with buffer. Electrophoresis was carried out in the Tiselius apparatus using a double section cell and an optical system of the type described by Philpot.[4] It proceeded for 80 minutes at a potential gradient of 8 volts per cm and a temperature of 1°C. Patterns were photographed directly, enlarged 5×, and the areas measured with a planimeter. Components were delineated by vertical lines drawn from the minima of the curves to the baseline. Ascending and descending pattern areas were averaged for each component except β-globulin; here only the ascending pattern area was used. Total and nonprotein nitrogen were determined by the micro-Kjeldahl method.

Results. In diffuse scleroderma, as shown in Table I, the albumin fraction of the serum proteins decreases and the γ-globulin fraction increases, but no significant change occurs in the total protein value. The alterations in the albumin and γ-globulin fractions are statistically significant. No new components in the electrophoretic pattern and no gross alterations in mobilities are evident. While little weight can be given to data from one case of localized scleroderma, it is interesting to note that the changes are qualitatively and quantitatively similar to those occurring in the diffuse scleroderma group. This is somewhat surprising in view of the apparent absence of systemic involvement in the localized form of the disease.

Discussion. The changes described are in no way diagnostic since similar changes occur in many chronic infections, in disseminated lupus erythematodes, in liver disease, and in other conditions.[5] They may, however, give

1 Matsui, S., *Press. med.*, 1924, **2**, 142.

2 Lindsay, J. R., Templeton, F. E., and Rothman, S., *J. A. M. A.*, 1943, **123**, 745.

3 Banks, B. M., *New Eng. J. Med.*, 1941, **225**, 433.

4 Philpot, J. St. L., *Nature*, 1938, **141**, 283.

5 Stern, K. G., and Reiner, M., *Yale J. Biol. and Med.*, 1946, **19**, 67.

TABLE I.
Electrophoretic Analysis of Serum Proteins in Scleroderma.

Patient No.	% of total protein					Concentration, g %					
	Alb.	Globulins				Alb.	Globulins				Total protein
		α_1	α_2	β	γ		α_1	α_2	β	γ	
					Diffuse Scleroderma.						
1	44.5	4.6	10.3	17.2	23.4	3.30	0.34	0.76	1.27	1.73	7.41
2	57.9	4.6	9.1	14.3	14.1	3.66	0.29	0.58	0.90	0.89	6.32
3	47.1	6.0	9.1	12.6	25.3	3.52	0.45	0.68	0.94	1.89	7.47
4	44.5	4.6	10.3	17.2	23.4	3.19	0.33	0.74	1.23	1.68	7.17
5	44.4	7.9	7.9	17.6	22.0	2.36	0.42	0.42	0.94	1.17	5.31
Mean	47.7	5.5	9.3	15.8	21.6	3.21	0.37	0.64	1.06	1.47	6.74
σ	5.82	1.45	1.00	2.22	4.38	0.508	0.067	0.140	0.178	0.422	0.921
					Localized Scleroderma.						
6	46.9	5.4	8.0	12.0	27.6	3.64	0.42	0.62	0.93	2.14	7.77
					Normal (12 subjects).						
Mean	58.4	4.7	9.2	15.2	12.8	4.00	0.32	0.63	1.05	0.89	6.90
σ	2.81	0.67	1.00	1.95	2.45	0.276	0.039	0.091	0.162	0.207	0.483

σ = Standard deviation.

TABLE II.
Clinical Data on the Patients.

Patient No.	Age	Sex	Duration of disease, yrs	Tissues involved in addition to skin	Course
1	25	F	3		Stationary
2	36	F	2	Joints	"
3	54	M	7	Esophagus, lungs, joints, pleura, pericardium, connective tissue (generalized)	Expired (autopsy)
4	58	M	2½	Joints	Expired
5	62	M	2	Esophagus, lungs, joints, renal and myocardial vessels*	" (autopsy)
6	13	F	6		Progressive

* Patient 5 also showed hypertension. Scleroderma was the only disease noted in any of the other patients.

some clue as to the pathogenesis of scleroderma. The rise in the γ-globulin fraction, which contains most of the known antibodies, suggests an immunological response, such as that which occurs during immunization,[6] or in the course of infectious diseases. Decrease in the albumin fraction, which occurs early in scleroderma, is also a characteristic of the electrophoretic pattern in malnutrition, liver disease and kidney disease.[5] Liver involvement is seldom found at autopsy in scleroderma, but kidney changes do occur, and a poor state of nutrition commonly accompanies the disease.

[6] Van der Scheer, J., Bohnel, E., Clark, F. H., and Wyckoff, R. W. G., *J. Immunol.*, 1942, **44**, 165.

16355 P

Effect of Acute Liver Damage on Ac-Globulin Activity of Plasma.*

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Normal plasma has been shown to contain a factor, Ac-globulin, which is a highly active accelerator of blood clotting.[1–3] It acts by catalyzing the interaction of prothrombin, thromboplastin and calcium with the resultant formation of thrombin. The hemorrhagic disease, parahemophilia, recently reported to occur in the human being,[4] is the result of a deficiency of this factor. Ac-globulin is differentiated from prothrombin in that it is precipitated from aqueous solutions at a higher pH, is more readily destroyed by heating, and is less soluble in concentrated ammonium sulfate solutions.[3,5] It is measured quantitatively[5] by an adaptation of the 2-stage method of prothrombin determination and may be detected in normal dog plasma in dilutions up to 350,000.

In acute liver damage, such as that produced by the administration of chloroform, there is a marked decrease in plasma prothrombin and fibrinogen.[6,7] In this paper we report that acute liver damage produced by chloroform also lowers plasma Ac-globulin.

Experimental. Quantitative estimation of Ac-globulin was achieved by an adaptation of the 2-stage prothrombin assay procedure. Details of the method are reported elsewhere.[5] In essence the method consists of comparing the rates of activation of prothrombin with thromboplastin and calcium ions in the presence of known and unknown amounts of Ac-globulin. The prothrombin, thromboplastin and calcium are kept constant. When prothrombin is activated in this manner under standard conditions the rate of thrombin formation is directly proportional to Ac-globulin concentration. Thrombin is measured by adding fibrinogen at intervals and noting the clotting time.

Three adult male dogs weighing 13 to 19 kg were observed for a period of 2 weeks and judged to be healthy on the basis of normal rectal temperature, maintenance of weight and food intake, alertness and activity. They were fasted for 36-72 hours and then given moderately deep chloroform inhalation anaesthesia varying in length of time from 1½ to 3 hours. Plasma Ac-globulin, coagulation time (Lee-White), hematocrit and icterus index were determined at intervals before and after chloroform administration. Prothrombin was determined by the 2-stage method of Warner, Brinkhous and Smith,[6,8] as modified by Ware and Seegers.[5] The modification consists only of supplying Ac-globulin when necessary. Heparin was used as the anticoagulant for blood specimens taken from one dog and 3.5% sodium citrate for those taken from the other 2 dogs.

Results. For 4 to 6 days following the administration of chloroform, the dogs showed lethargy, anorexia, and a low-grade temperature increase. The establishment of liver damage was shown by the development of an icteric color to the plasma first becoming noticeable 24-48 hours after the anaesthesia, and by the development of hypoprothrombinemia. At no time, however, did the animals show clinical jaundice or clay-colored stools.

* Aided by a grant from the U. S. Public Health Service.

1 Owren, P. A., *Proc. Norwegian Acad. Science,* Nov. 10, 1944, p. 21.

2 Fantl, P., and Nance, M., *Nature,* 1946, **158**, 708.

3 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.,* 1947, **169**, 231.

4 Owren, P. A., *Lancet,* 1947, **252**, 446.

5 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.,* 1948, **172**, 699.

6 Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.,* 1937, **66**, 801.

7 Foster, D. P., and Whipple, G. H., *Am. J. Physiol.,* 1922, **58**, 407.

8 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.,* 1936, **114**, 667.

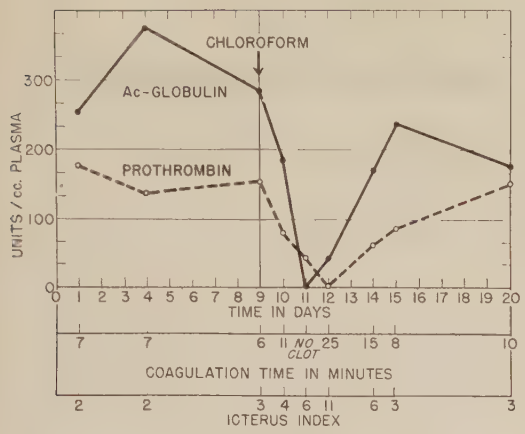

Fig. 1.

The effect of acute liver damage (chloroform intoxication) on the plasma Ac-globulin concentration of the dog.

The onset of the plasma jaundice and the peak of the icterus index rise were not associated with a drop in the hematocrit. All of the dogs survived.

The duration of chloroform administration, from 1½ to 3 hours, appeared to have no marked influence on the resulting changes in Ac-globulin and prothrombin concentrations. The values shown in Fig. 1 for the dog anesthetized for a period of 3 hours are representative for the group. Within 24 to 48 hours after anaesthesia, the plasma Ac-globulin decreased precipitously in all dogs from initial values of 100 to 370 units per cc to low values of 1 to 3 units per cc. Prothrombin also decreased to low levels but did so somewhat later, within 48 to 72 hours after anaesthesia. Ac-globulin returned to normal values in approximately 6 days, while prothrombin returned more slowly, reaching normal values in about 10 days. Significantly increased coagulation time, hematoma formation and excessive bleeding from venipuncture wounds were encountered 2 days after anaesthesia; the dog receiving 3 hours of anaesthesia, in addition, showed continuation of the bleeding tendency through the third day. Fibrinogen was absent in this dog 2 days after anaesthesia as shown by the failure of blood samples to clot following the addition of a very active preparation of thrombin.

Discussion. These experiments indicate that the hemorrhagic tendency occurring in cases of acute liver damage is attributable not only to decreased prothrombin and fibrinogen concentration[6,7] but also to decreased plasma Ac-globulin. The data suggest further that plasma Ac-globulin is formed in the liver.

Summary. Acute hepatic damage in the dog, caused by chloroform intoxication, is followed by a pronounced and rapid decrease in plasma Ac-globulin concentration. This decrease is accompanied by a parallel decrease in plasma prothrombin concentration. Ac-globulin returns to normal values more quickly than does prothrombin.

16356

Prevention by Sodium Salicylate of Arteritis in the Experimental Allergic State.*

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It has been established that arteritis can be produced in the experimental animal following the injection of foreign protein.[1–6] We have attempted to produce experimental arteritis in one group of rabbits and to prevent its expected production in a similar group, with the purpose of clarifying the mechanism of its pathogenesis. Several extensive reviews of the problem have appeared, among which is that of Logue and Mullins.[7] The pathology has been described, and circulating antibody has been studied.[1,4,6,8,9,10,11] The process which precedes antigen-antibody reaction in the fixed tissues has in part been indirectly demonstrated[12–15] by the recovery of humoral antibody against homologous kidney from animals injected with hemolytic streptococci; this suggests initial union of the streptococcus antigen with a kidney tissue hapten fraction. A histologic correlation with the immunological process in experimental arteritis[6] has shown that one of the earliest manifestations of the lesion is collection of lymphocytes and monocytes around the artery at which time circulating antibody is absent. These observers[6] present these findings as evidence of fixation of antigen in the arterial wall, and of antibody migration within the lymphocytes to the site of fixed antigen.

Sodium salicylate suppresses circulating antibody in the experimental animal[1,16,17] and in clinical serum sickness.[9] Given intravenously[1] it did not prevent the occurrence of experimental arterial lesions. Suppression of circulating antibody was reconciled with hastening of recovery by postulating that the same mechanism which rendered an infectious agent less antigenic might also render it less pathogenic.[16] Sodium salicylate *in vitro* exerts some inhibitory effect on immune reactions.[18,19] Some investigators have found

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1 Hagebush, O. E., and Kinsella, R. A., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 922.

2 Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, **72**, 65.

3 Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 239.

4 Fox, R. A., and Jones, L. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 294.

5 Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. Clin. Med.*, 1947, **32**, 379.

6 Hawn, C. van Z., and Janeway, C. A., *J. Exp. Med.*, 1947, **85**, 571.

7 Logue, R. B., and Mullins, F., *Ann. Int. Med.*, 1946, **24**, 11.

8 Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, **27**, 341.

9 Derick, C. L., Hitchcock, C. H., and Swift, H. F., *J. Clin. Invest.*, 1928, **5**, 427.

10 Mackenzie, G. M., and Leake, W. H., *J. Exp. Med.*, 1921, **33**, 601.

11 Fleisher, M. S., and Jones, L. R., *J. Exp. Med.*, 1931, **54**, 597.

12 Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **39**, 148.

13 Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 158.

14 Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 163.

15 Jones, L. R., personal communication.

16 Swift, H. F., *J. Exp. Med.*, 1922, **36**, 735.

17 Homburger, F., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 101.

18 Marrack, J., and Smith, F. C., *Brit. J. Exp. Path.*, 1931, **12**, 30.

19 Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, **77**, 173.

20 Guerra, F., *Science*, 1946, **103**, 686.

in vitro and *in vivo* inhibition of hyaluronidase activity by salicylates[20,21] and believe this is due to inhibition of the enzyme reaction involved in liberation of N-acetyl-glucosamine. The toxic effects of salicylates have been studied[22,23,24] and are proportional to size of dosage.

Anti-histaminic drugs protect against anaphylaxis[25] but there is no correlation between anaphylactic reactions and the occurrence of vascular lesions.[4] However, the anti-histaminic drugs greatly minimize the vascular lesions in experimental arteritis[5] and do have an anti-hyaluronidase action.[26] Hyaluronidase greatly intensifies the size of epidermal allergic reactions.[26]

Para-aminobenzoic acid is apparently concerned with metabolic stimulation of host cells.[27]

Method. Twenty albino rabbits were given intravenous injections of sterile horse serum on 3 occasions. The first dose was 10 cc per kilo; the second dose, given 16 days later, was one cc per animal; the third dose, given 6 days later, was 10 cc per kilo. Seven days after the third injection all surviving rabbits were sacrificed by air emboli and were immediately autopsied. The organs were fixed in formalin, sectioned, stained, and the heart and kidneys carefully studied for the presence of arteritis.

The animals were divided into 4 groups.

Group A, 3 rabbits, was given para-aminobenzoic acid starting 31 days prior to the first injection of horse serum and continuing throughout the course of the experiment. The PABA was dissolved in their drinking water, each cc containing 0.0026 g of PABA.

Group B, 12 animals, was given sodium salicylate daily, starting 8 days prior to the first injection of horse serum and continuing throughout the course of the experiment. A 5% aqueous solution was employed and the injections were given subcutaneously once each day. The daily dose was 0.2 g per kilo of body weight.

Group C, 2 animals, was treated exactly the same as Group B with the exception that each injection of sodium salicylate was given intravenously prior to the first injection of horse serum. After that all sodium salicylate injections were given subcutaneously.

Group D, 3 animals, received nothing other than horse serum.

The animals in all groups were bled on one or several occasions and the serum was tested for the presence of precipitin against horse serum.

Results. The results are shown in Table I. The 3 animals in Group B that failed to survive died during the night in the last week of the experiment. In the presence of postmortem changes no conclusions can be drawn from the appearance of their tissues. However all 3 showed the same findings, consisting of gangrene of the caecum, peritonitis, and lymphocytic and monocytic infiltration around the arteries of the kidneys. Group C animals both showed, in addition to periarterial round cell infiltration, diffuse and localized collections of lymphocytes throughout the parenchyma of the kidney, thickening of some glomerular membranes, and infiltrations of mononuclear cells around the afferent arteriole.

Discussion. Whereas the horse serum control animals and 2 of the PABA treated animals developed arterial lesions and circulating antibody at the end of the experiment, the findings were different in the sodium salicylate treated animals. Moreover, the route of administration of the sodium salicylate apparently influenced the effect. When it was given subcutaneously the surviving animals showed no evidence of arteritis but did produce circulating antibody in moderate or attenuated quantity, late in the course of the experiment.

21 Dorfman, A., Reimers, E. J., and Ott, M. L., 1947, **64**, 357.

22 Stevenson, C. S., *Am. J. Med. Science,* 1937, **193**, 772.

23 Guest, G. M., Rapoport, S., and Roscoe, C., *J. Clin. Invest.,* 1945, **24**, 770.

24 Jager, B. V., and Alway, R., *Am. J. Med. Science,* 1946, **211**, 273.

25 Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science,* 1945, **102**, 93.

26 Mayer, R. L., and Kull, F. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 392.

27 Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.,* 1944, **80**, 561.

TABLE I.

Effect of PABA and of Sodium Salicylate and Its Route of Administration on Humoral Antibody and Periarterial Lesions in Rabbits Injected with Horse Serum.

Rabbit No.	PABA, oral	Sodium salicylate, subcut.	Sodium salicylate, I.V. and subcut.	Precipitin reaction: 12 days after first Horse serum	Precipitin reaction: Day of second Horse serum	Precipitin reaction: Day of third Horse serum	Precipitin reaction: Last day of exper.	Died during exper.	Periarterial lesions
1.	Yes						1:1280		Negative
2.	,,						1:1280		Positive
3.	,,						1:1280		,,
4.		Yes		Neg.	Neg.	Neg.	1:140		Negative
5.		,,		,,	,,	,,	1:160		,,
6.		,,		,,	,,	,,	1:200		,,
7.		,,			,,	,,		Yes	?
8.		,,			,,	,,	1:120		Negative
9.		,,			,,	,,	1:200		,,
10.		,,			,,	,,		,,	?
11.		,,			,,	,,	Neg.		Negative
12.		,,			,,	,,		,,	?
13.		,,			,,	,,	1:120		Negative
14.		,,			,,	,,	1:80		,,
15.		,,			1:180	,,	1:160		,,
16.			Yes	,,	Neg.	,,	1:6		Positive
17.			,,	,,	,,	,,	1:16		,,
18.							1:1280		,,
19.							1:640		,,
20.							1:640		,,

When it was given intravenously first and subcutaneously later the animals did develop arteritis and glomerular changes, but produced circulating antibody in scarcely detectable quantity, late in the course of the experiment.

The following theory is offered as a possible explanation of the reactions here observed. When sodium salicylate is administered subcutaneously sufficiently in advance of the first injection of antigen, it blocks the fixation of antigen in the tissue cells. This precludes any need of antibody in the tissues and there is, therefore, no lymphocytic or monocytic migration to the tissues. In this absence of tissue antigen-antibody union the chain of events leading to production of lesions is never started. The intravenously injected antigen circulates for a prolonged period, and in its passage through the lymph nodes and other reticulo-endothelial structures gives rise to production of antibody. Union of this circulating antibody with its antigen is prevented by reason of the inability of antigen to become fixed to tissue cells. The presence of this circulating antibody thus becomes an indifferent matter to the well being of the host, at least insofar as this type of allergic reaction is concerned. The observations of others concerning the potentiating effect of hyaluronidase in a certain type of allergic reaction[26] and the anti-hyaluronidase activity of sodium salicylate,[20,21] suggest that sodium salicylate blocks antigen fixation in the tissues by inhibiting hyaluronidase activity.

No conclusions can be drawn concerning the effect produced when sodium salicylate is given intravenously prior to the first injection of the antigen and subcutaneously thereafter, since only 2 animals were treated in this manner. However, the identical findings in both suggest a lowering of the protective action against antigen-tissue fixation, and a heightening of the depressant effect on the reticulo-endothelial system. This problem is at present under further investigation.

Conclusions. 1. The subcutaneous administration of large doses of sodium salicylate to rabbits well in advance of the initial contact with horse serum antigen prevents the development of arterial lesions.

2. The arterial lesions fail to develop even though circulating antibody is present in moderate quantity.

3. It is believed that the lesions fail to develop because there is no antigen-antibody reaction, and that this reaction can not take place because salicylate has prevented antigen from uniting with tissue cells.

16357

Specificity of the Response of Various Assay Organisms to Nicotinic Acid.*

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It has recently been shown with *Neurospora* mutants that tryptophan can be converted to nicotinic acid and that kynurenine and hydroxyanthranilic acid are intermediates in the conversion.[1,2] This conversion, which also appears to be carried out by the intestinal flora of animals,[3,4] and possibly by animal tis-

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1 Beadle, G. W., Mitchell, H. K., and Nyc, J. F., *Proc. Nat. Acad. Sci.*, 1947, **33**, 155.

2 Mitchell, H. K., and Nyc, J. F., *Proc. Nat. Acad. Sci.*, 1948, **34**, 1.

3 Ellinger, P., and Abdel Kader, M. M., *Nature*, 1947, **160**, 675.

sues,‡ is of considerable interest in human nutrition.[5] Since microörganisms are widely used for the determination of nicotinic acid, and since these intermediates occur naturally under some conditions, it was of interest to determine whether the commonly used assay organisms could utilize *l*-kynurenine or hydroxyanthranilic acid for growth in place of nicotinic acid. For the organisms tested, these compounds were uniformly inactive. The data essential for establishing this fact are presented below.

Experimental. Cultures and Methods. The cultures used were *Lactobacillus arabinosus* 17-5, *Leuconostoc mesenteroides* P-60, *Streptococcus faecalis* R, *Proteus vulgaris* and *Torula cremoris* 2512. The niacin assay medium employed with the first three organisms was the complete amino acid assay medium described by Henderson and Snell,[6] with niacin omitted. For *Proteus vulgaris,* the medium of Saunders *et al.*[7] was used. With *Torula cremoris,* the medium and assay conditions described by Williams[8] were employed. In all cases, solutions of *l*-kynurenine and hydroxyanthranilic acid§ were sterilized by filtration, and added aseptically to the basal media after these had been sterilized by autoclaving. The final volume in all assay tubes was 2 cc. After a three-day incubation period, which permitted heavy growth of all organisms except *Proteus vulgaris* in media containing excess niacin, cultures were diluted

TABLE I.

Growth Response of Various Organisms to *l*-Kynurenine and Hydroxyanthranilic Acid in Presence and Absence of Nicotinic Acid.*

Substance added γ per 2 cc	Nicotinic acid			*l*-Kynurenine		Hydroxyanthranilic acid		Hydroxyanthranilic acid plus kynurenine 0.5 γ each	Niacin (.05 γ) plus hydroxyanthranilic acid (0.5 γ) plus kynurenine (0.5 γ)
Organism	0	.05	10	0.1	10	0.1	10	% of incident of light transmitted (diluted cultures)	
L. arabinosus	91	70	43	91	97	95	94	95	74
Leuc. mesenteroides	95	74	60	95	96	96	96	98	76
S. faecalis	92	79	82	92	92	92	92	92	80
P. vulgaris	99	92	—	99	99	99	99	99	92
T. cremoris	99	77	53	99	99	97	99	96	76

* Several levels of each substance in addition to those shown were tested. In no case was evidence of any growth-promoting action apparent.

4 Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.,* 1946, **163**, 343.

‡ *Cf.* the synthesis of niacin which occurs during incubation of hen (Quarles, E., and Snell, E. E., *J. Nutrition,* 1941, **22**, 483) and turkey (Furman, C., Snell, E. E., and Cravens, W. W., *Poultry Science,* 1947, **26**, 307) eggs, where no microörganisms are present.

5 Anonymous, *Nutrition Rev.,* 1947, **5**, 247.

6 Henderson, L. M., and Snell, E. E., *J. Biol. Chem.,* 1948, **172**, 15.

7 Saunders, F., Dorfman, A., and Koser, S. A., *J. Biol. Chem.,* 1941, **138**, 69.

8 Williams, W. L., *J. Biol. Chem.,* 1946, **166**, 397.

§ We are indebted to Dr. H. K. Mitchell for samples of *l*-kynurenine and hydroxyanthranilic acid. Synthesis of the latter compound has been described recently (Nye, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.,* in press).

with water to 10 cc and turbidity values determined with the Evelyn photoelectric colorimeter. Results are given in Table I. In no case did kynurenine or hydroxyanthranilic acid, either singly or combined, in the presence or absence of sub-optimal quantities of niacin, enhance growth. Ornithine, glutamine and arginine, which increase nicotinamide production by some strains of *E. coli*, have also been shown to be ineffective in substituting for the niacin requirement of *L. arabinosus*,[3] the organism most commonly used for assay of this vitamin.

Summary. Kynurenine and hydroxyanthranilic acid, which appear to be intermediates in the conversion of tryptophan to nicotinic acid by *Neurospora*, neither replace nicotinic acid nor enhance the growth response to sub-optimal amounts of nicotinic acid for *L. arabinosus, Leuc. mesenteroides, S. faecalis, Proteus vulgaris*, or *Torula cremoris*. These substances do not, therefore, interfere in microbiological assays for niacin which employ these test organisms.

Appreciation is expressed to Professor Ch. Weizmann whose kindness made this study possible.

16358

Paper Partition Chromatographic Analysis and Microbial Growth Factors: The Vitamin B_6 Group.

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Microbial growth factors often exist in nature in more than one chemically defined form. In the past, differential assays with several microörganisms have been used to demonstrate this multiplicity.

The present report deals with an alternate procedure for recognizing the presence of different forms of a growth factor. The method involves the combined use of paper partition chromatography and a microbial indicator and represents, as far as the writers are aware, a new methodological principle for the recognition and resolution of different chemical forms of a growth factor.

As will be seen, the method to be described is the counterpart of the technique used in the differention of the several forms of penicillin by Goodall and Levi[1] and independently by Winsten and Spark[2] except that in dealing with growth factors, zones of microbial growth serve to delineate the different members of a complex instead of zones of inhibition, as in the case of the antibiotics. The use of zones of microbial growth to assay vitamins was suggested by the work of Mager and Aschner.[3]

In the present method, the underlying principle involves the separation on a paper strip chromatogram of the different chemical forms of a growth factor. After the separation has been achieved, the positions of the different forms on the paper strip are revealed by the use of a microbial indicator, that is, a microörganism capable of utilizing at least one and usually several forms of the factor for growth. The positions of the various chemical entities on the chromatogram serve to characterize and identify the particular substances present. This is a consequence of the fact that the distance each substance moves is related to its specific partition coefficient for the solvents employed in developing the chromatogram.

In what follows, the application of this method to the resolution and identification of the members of the B_6 group will be described. This particular complex of growth factors was

1 Goodall, R. R., and Levi, A. A., *Nature*, 1946, **158**, 675.

2 Winsten, W. A., and Spark, A. H., *Science*, 1947, **106**, 192.

3 Mager, J., and Aschner, M., *J. Bact.*, 1947, **53**, 283.

studied first since its component parts are well known, principally due to the work of Snell.[4] The present report is intended to demonstrate the potentialities of the technique employed and to describe some results obtained by its use.

Method. In applying the method to the vitamin B_6 group, a 0.007 ml drop of a solution (pH adjusted to 5.0) containing 1-5 μg per ml of each of the various members of the group is applied near the head of a paper strip chromatogram (Whatman No. 4 paper strips 1″ by 16″ are used). The chromatogram is then developed for about 6 hours with wet butanol in a humid atmosphere in the manner first devised for amino acids by Consden, Gordon and Martin.[5] After drying for 15 minutes at 65°C, the paper strip chromatogram with the various members of the vitamin B_6 complex now occupying definite positions along the strip, is laid on an agar plate, seeded with *Saccharomyces carlsbergensis,* strain 4228, an organism which exhibits a growth response to all three known forms of the vitamin. The nutrient agar[6] contains all factors necessary for the growth of the organism with the exception of the vitamin B_6 group. In making the agar plate a bottom layer of the nutrient agar is made by pouring 300 ml of the agar medium on a plate 11″ by 18″. This is allowed to harden. A 200 ml portion of the nutrient agar cooled to 48-50° C is seeded with 10 ml of sterile physiological saline to which has been added a loopful of a 24-hour culture of the yeast. The seeded agar is then poured on the hardened underlayer and allowed to cool. (Using such a plate, as many as 8 strip chromatograms resulting from eight separate analyses may be laid parallel to each other on the nutrient agar at one time). The paper strip chromatogram is allowed to soak for 5 minutes on the surface of the moist agar in order to transfer the various B_6 members from the strip to the agar plate. The strip is then removed leaving its imprint, however, on the agar surface. The agar plate is then incubated overnight at 27-30°C. After incubation well defined elliptical zones of growth of the organism are seen at various intervals along the locus of the chromatogram marking the loci of the various members of the vitamin B_6 group. The area of each zone of growth is a measure of the amount of the particular substance causing the growth. While it has proved possible to obtain a dose response curve for each substance by plotting area of growth against weight of substance per ml, the greatest utility of the method lies in its ability to resolve and identify the various members in complex mixtures of the different forms of vitamin B_6. Visual inspection of the size of the zones of growth gives a graphic picture of the relative amounts of the various forms present in a mixture.

The different members of a complex are identified by their relative positions along the length of a chromatogram. Each substance has its characteristic R_F value which is defined according to Consden *et al.*[5] as the ratio of the distance a substance has moved down the chromatogram to the distance the developing solvent has traveled. This is conveniently measured directly on the agar plate if one imprints on the agar the spot where the original drop was applied as well as the boundary on the strip to which the solvent front has moved. This is readily done when the paper strip is still in place on the agar surface. The R_F value is then equal to the distance of the center of a zone of growth from the point of application of the sample being analyzed, divided by the distance the solvent has moved measured from the point of application of the drop.

Results. The above technique was first applied to the resolution of the members of the vitamin B_6 group in a synthetic mixture.

On chromatographing a 0.007 ml sample of a mixture containing 5 micrograms each per ml of pyridoxal, pyridoxine and pyridoxamine with wet butanol, the characterizing R_F values for the component factors were found to be: pyridoxamine 0.18, pyridoxal

[4] Snell, E. E., *J. Am. Chem. Soc.*, 1944, **66**, 2082.

[5] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

[6] Atkin, L., Schultz, A. S., Williams, S. L., and Frey, L. M., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

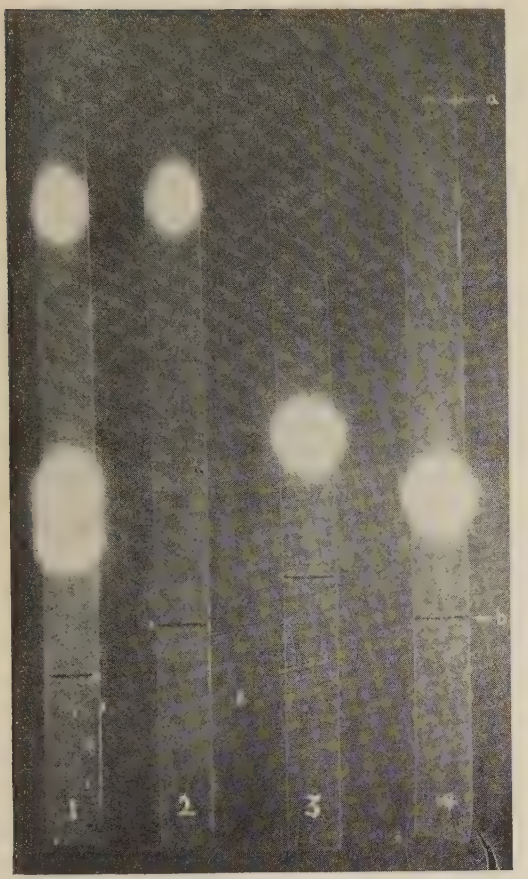

FIG. 1.
Results of paper partition chromatography applied to synthetic members of the B_6 group. The photograph represents a picture of a typical agar plate and shows the zones of growth of *S. carlsbergensis*, Strain No. 4228, along the loci of the paper strip chromatograms. The symbol (a) refers to the locus of point of application of the sample. The symbol (b) refers to the boundary to which the solvent had moved. The strips represent the result of chromatographing a mixture of pyridoxal, pyridoxamine, and pyridoxine (strip 1), pyridoxamine alone (strip 2), and pyridoxal alone (strip 3), and pyridoxine alone (strip 4).

0.68, pyridoxine 0.75. That is, pyridoxamine moves least rapidly on the chromatogram. Fig. 1 shows the results of such an experiment.

The R_F values were found to vary 10 to 20% from day to day. However, on any given day replicate chromatograms gave self-consistent R_F values. The relative positions on the chromatograms of the different forms of B_6 remained the same.

In order to simulate what might occur on chromatographing an extract of the vitamin B_6 group obtained by the usual acid hydrolysis of natural products, a study was then made of the influence of a mixture of amino acids on the R_F values of the B_6 group members since various amino acids might be expected to be present in such extracts. Accordingly, the various forms of B_6 dissolved in a solution containing 5 per cent casein hydrolysate adjusted to pH 5, were chromatographed as above.

The presence of the amino acids caused a diminution in the R_F value of pyridoxamine from 0.18 to 0.06; that for pyridoxine decreased from 0.75 to 0.64.

Since the presence of amino acids in solutions being analyzed for the B_6 group alters the R_F values, these are not sufficient to identify the B_6 components unequivocally in an unknown mixture. It has been found expedient to identify members of the B_6 group in an extract of natural origin by obtaining a second chromatogram of the sample to which a known form of B_6 has been added to serve as a marker. The relative position occupied by the various B_6 members to that of the known form of B_6 added as a marker, serve to identify the former.

The results obtained on chromatographing a solution of pyridoxal and casein hydrolysate amino acids indicated that pyridoxal had reacted with certain constituents of the hydrolysate. Two zones of growth were observed. One was identified as pyridoxamine. On standing for several days at icebox temperature under aseptic conditions the pyridoxamine zone increased in size indicating that almost half the original pyridoxal was converted to pyridoxamine. Snell and Rannefeld[7] have shown that on heating pyridoxal in solutions of amino acids, it is converted to pyridoxamine. However, the ease with which this reaction occurs, even at low temperatures, has not been fully appreciated. This finding throws some doubt on the validity of the usual type of microbiological assay for pyridoxal where the extract also contains amino acids, since these might convert an unknown frac-

[7] Snell, E. E., and Rannefeld, A. H., *J. Biol. Chem.*, 1945, **157**, 475.

tion of pyridoxal to pyridoxamine.

The second zone observed on chromatographing pyridoxal in casein hydrolysate solution, namely that due to pyridoxal itself, was found to be more diffuse and cover a larger area along the locus of the chromatogram. This lengthening of the zone was interpreted as being due to a reversible reaction of pyridoxal and certain amino acids, a fact suspected by Snell but not heretofore easily demonstrated.

In using the analytical technique under discussion it has been possible to demonstrate a heretofore unrecognized reaction of a reversible type between *L*-cysteine and pyridoxal. This reaction which is non-enzymatic, occurs merely on incubating together a concentrated solution of cysteine and pyridoxal. The reaction is to be distinguished from that occurring between pyridoxal and other amino acids such as glutamic acid to yield pyridoxamine. In addition to a small but variable amount of pyridoxamine which does form during the reaction, there is an additional substance formed which moves more slowly on the chromatogram than does pyridoxamine. A typical experiment in which pyridoxal and cysteine were allowed to react was conducted as follows: 179 mg of pyridoxal hydrochloride and 157 mg of cysteine hydrochloride were dissolved in one ml of water. After standing overnight at 37°C the solution was diluted to 5 ml and 0.16 ml of pyridine was added to neutralize the acidity. The solution was then allowed to stand 2 days at room temperature. After appropriate dilution to yield solutions containing the nominal amount of 360 μg per ml and 36 μg per ml of pyridoxal hydrochloride based on the original amount of pyridoxal hydrochloride used, and adjustment to pH 5.0, 0.007 ml samples were taken for chromatographic analysis. The results of this analysis appear in Fig. 2. An examination of the replicate chromatograms 3 and 4 obtained for the more concentrated sample, reveals two zones of growth connected by a band of growth. The lower zone is due to the faster moving unchanged pyridoxal. The upper zone is due to a new substance which moves on the chromatogram at a rate less than that of

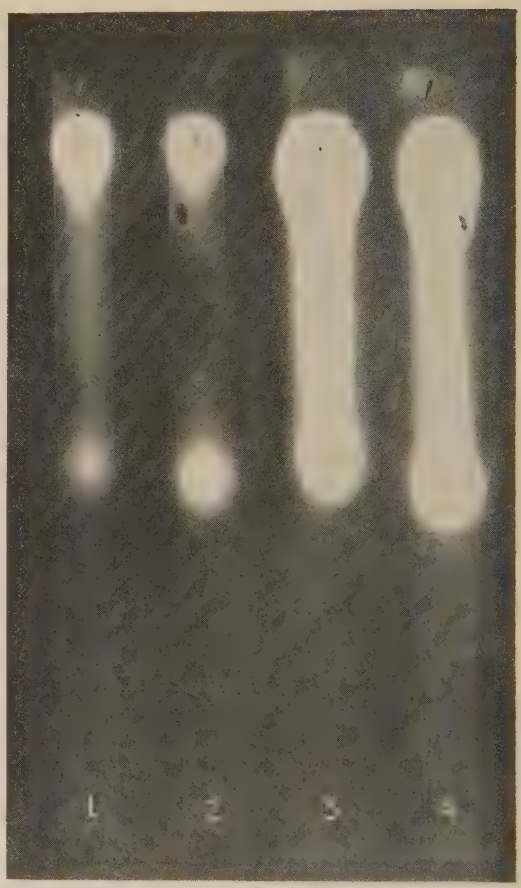

FIG. 2.
Results of paper chromatographic analysis of a reaction mixture of *l*-cysteine and pyridoxal. Duplicate strips 1 and 2 represent the analyses of a 0.007 ml sample of a solution containing the equivalent of 36 micrograms of pyridoxal hydrochloride per ml. Duplicate strips 3 and 4 are those obtained for a solution containing the equivalent of 360 μg per ml of pyridoxal hydrochloride.

pyridoxamine itself as established in a separate experiment. The new zone of growth probably represents an adduct formed from cysteine and pyridoxal and is either a new form of vitamin B_6 or a substance which readily regenerates the original pyridoxal for use by the microörganism. The reaction to yield the substance in question is evidently reversible. Such reversibility would explain the band of growth connecting the two zones since, as the solvent used to develop the chromatogram moves the pyridoxal down the column, its removal from the original point of application of the sample will reverse

the equilibrium reaction of cysteine and pyridoxal regenerating the latter; this would then make its appearance known by causing a tail of growth on the main pyridoxal zone as observed experimentally.

Examination of the replicate chromatograms 1 and 2 (Fig. 2) of the more dilute sample again reveals 2 zones of growth, one due to a new substance moving more slowly than pyridoxamine, and the other moving like pyridoxal itself. There is little trace seen of the band of growth connecting the zones as observed in analyzing the more concentrated sample. This result suggests the formation of a second more stable derivative of pyridoxal and cysteine whose existence is not so readily disturbed by dilution and which moves at about the same rate as the unstable unknown substance whose existence was noted in the more concentrated sample. As a consequence, for the more concentrated sample only one composite zone is seen at the top of the chromatogram and may be due as indicated above to two substances.

The reaction of pyridoxal and cysteine may take place in a manner similar to that of other simple aldehydes and aldoses with cysteine as demonstrated by Schubert.[8] Thus a simple adduct may form between the cysteine sulfhydryl group and pyridoxal. This reaction might be expected to be quite reversible. The adduct may then cyclize to give a thiazolidine ring derivative of vitamin B_6. The latter compound would probably be more stable to hydrolysis than the 1st adduct mentioned. This compound might represent the slow moving substance observed on chromatographing the more dilute sample.

The compounds formed from pyridoxal and cysteine for which evidence has been presented above, may represent new forms of vitamin B_6 of physiological interest hitherto unrecognized because of their unstable character.

It might have been quite difficult to demonstrate such a reaction between cysteine and pyridoxal with the usual form of microbiological assay.

A further result of practical interest is the finding that many preparations of synthetic nature such as pyridoxal itself and pyridoxal phosphate are impure and contain foreign substances which may themselves be active as growth substances replacing B_6. Thus the present technique provides a delicate test of purity for standard preparations of growth factors used in biological and biochemical investigations.

Studies are now being conducted on natural products using this method. These will be reported at a later date.

The potential usefulness of the general technique involving a combination of paper partition chromatography and microbiological indicators should be of value in studies on the differentiation of other growth factors such as folic acid which occur in nature in more than one chemically defined form. This consideration also prompted the present report.

Summary. 1. A new technique of resolving and identifying the members of the B_6 family of growth factors is described. It involves the use of paper partition chromatography coupled with a microbial indicator.

2. Using this method it has been demonstrated that pyridoxal undergoes a non-enzymatic transamination reaction even in the cold. Pyridoxal also appears to react reversibly with other amino acids.

3. The reaction between cysteine and pyridoxal differs from that with other amino acids in that one can demonstrate the formation of at least one adduct which moves more slowly on the chromatogram than does pyridoxamine. This reaction may produce a new form of vitamin B_6 which may also have a transient existence in nature.

4. The technique described should prove of value as a means of establishing the purity of the various forms of vitamin B_6 used in biological and biochemical studies.

5. The general technique employing paper partition chromatography and microbial indicators should be of value in resolving other multiform vitamins into their constituent parts.

[8] Schubert, M., *J. Biol. Chem.*, 1939, **130**, 601.

·16359

Influence of Hemorrhage and of Albumin Injections upon the Sodium Concentration of Human Plasma.*†

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The purpose of this paper is to present data on the change in concentration of plasma sodium following phlebotomy in 4 normal subjects as well as on the changes in plasma sodium concentration after injections of salt-poor human albumin and/or concentrated plasma in several patients with malnutrition and hypoalbuminemia. The latter were part of a larger group in which other effects of administering salt-poor albumin and concentrated plasma were studied and reported.[1]

Methods. Control blood samples were drawn without stasis from the antecubital vein with the subject in the supine position. All succeeding specimens were obtained from the opposite arm. Clotting was prevented by moistening the barrel of the syringe with a small amount of a commercial heparin solution. After thorough mixing the samples were transferred to hematocrit tubes 8 mm in diameter and centrifuged at 3,000 r.p.m. for 40 minutes, after which the supernatant plasma was carefully removed. One cc of the plasma thus obtained was diluted to 100 cc and thoroughly mixed. A Perkin-Elmer flame photometer was used for making the sodium determinations. A standard solution for calibration contained 50 parts of sodium, as sodium chloride, per million (50 mg per liter) in distilled water. This solution read 100 on the machine. Intermediate values were obtained with standard solutions of concentrations varying from 0 to 50 mg per liter. The calibration curve deviated only slightly from a straight line. Readings of the unknown samples were recorded and corresponding sodium concentrations were determined from the curve. Frequent resettings of the zero and standard were made while performing each determination. Five readings were recorded for each sample and the average used as the most accurate value. The maximum range noted in the 5 readings was about 1.5 scale divisions, the average deviation ± 1 division, equivalent to approximately ± 1 milliequivalent of sodium. The flame photometer determinations on 3 samples of plasma were checked by gravimetric methods. Agreement within 2 milliequivalents was obtained. For a complete discussion of use and limitations of the flame photometer for such purpose the reader is referred to the paper of Held.[2]

Findings. Sodium concentrations, determined in 7 normal males 23 to 49 years of age, at intervals of 5, 10, 15, 20, 60, 120, and 180 minutes, showed little variation. The concentrations ranged from 143 to 157 milliequivalents per liter in the several subjects. The extreme differences noted in the 2 to 6 successive samples drawn from the same subject were only 1 to 5 milliequivalents per liter. The average normal value of 147 milliequivalents per liter is in close agreement with the figure of 144 milliequivalents per liter reported by Marinis, Muirhead, Jones and Hill.[3]

Effect of Phlebotomy. In 4 normal young men the plasma sodium concentration was measured before, immediately after and at intervals of ½, 1, 2, 3, and 24 hours after rapid removal of 500 cc of blood. The sodium concentration fell significantly in each instance. These data are recorded in Fig. 1. In one subject the plasma potassium was simul-

* The serum albumin used in this study was processed by the American National Red Cross from blood which it collected from volunteers.

† Aided by a grant from the Commonwealth Fund.

1 Elman, R., Kelly, F. J., and Simonsen, D. H., in press.

2 Held, P. M., *J. Biol. Chem.*, 1947, **167**, 499.

3 Marinis, T. P., Muirhead, E. E., Jones, F., and Hill, J. M., *J. Lab. and Clin. Med.*, 1947, **32**, 1208.

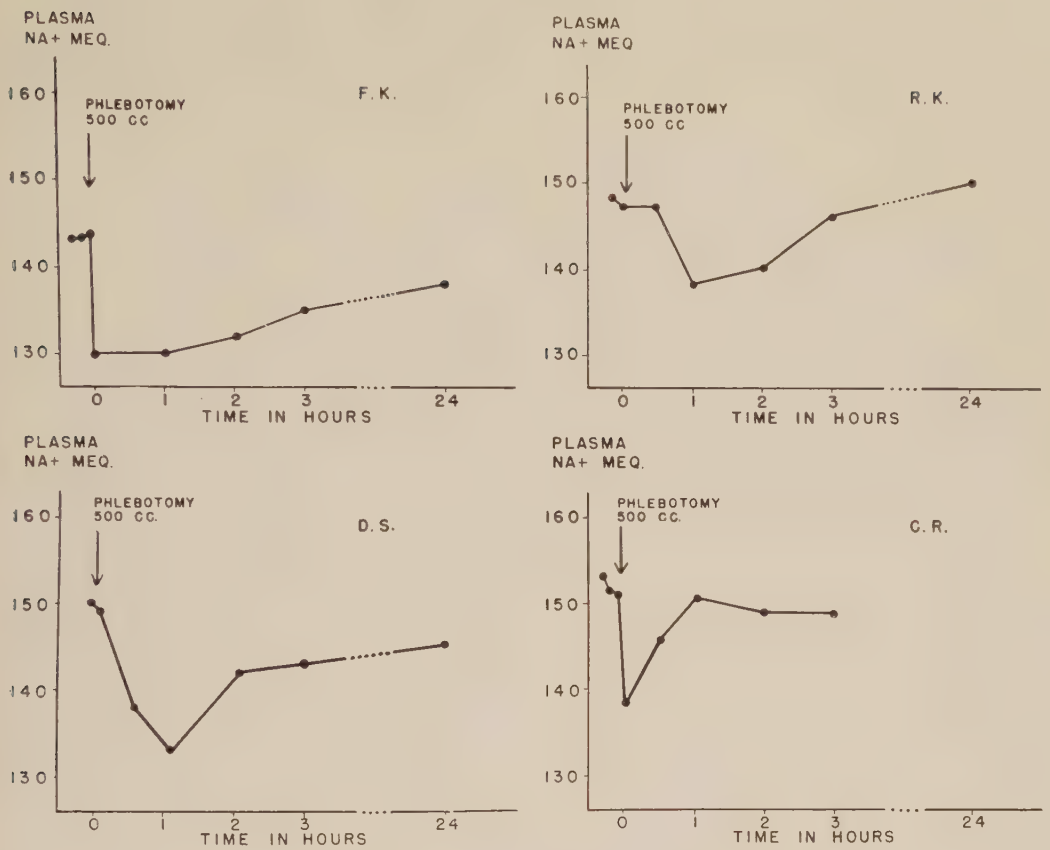

FIG. 1.

Fall in Serum Sodium Following Phlebotomy.

The 4 curves shown above represent observations on 4 healthy donors bled 500 cc. Note the constant drop in the plasma sodium concentration with its return to normal varying from 1 to 3 or more hours.

taneously determined, but no change was noted.

Effect of Injection of Albumin and of Plasma. In 5 patients having malnutrition, hypoalbuminemia and edema, the plasma sodium concentration was measured before, immediately after, and at intervals of 1, 3, 6, and 24 hours after the infusion of either 25% salt-poor human albumin or concentrated plasma.‡ (Table I) Four patients were given 7 injections of albumin; 1 patient received albumin on one occasion and concentrated plasma 2 days later; one patient received concentrated plasma alone. Following 5 of the 7 albumin injections, the plasma sodium concentration fell significantly, the maximum decrease occurring immediately after infusion (at which time the plasma volume showed its maximum increase).[2] In one case (B.P., Table I) in which plasma potassium concentration was simultaneously determined, no significant change was found. In the 2 patients receiving concentrated plasma the plasma sodium content increased significantly, the highest value being obtained 3 hours after injection in both. Immediately after plasma infusion the plasma volume was increased maximally, as with albumin, and at this time the plasma sodium concentration had changed little, or not at all.

‡ Two 500 cc units of Red Cross lyophilized plasma were dissolved in 1 unit (500 cc) of distilled water. The sodium content was determined by us to be 3.80 g per 500 cc; that of the 25% albumin solution was recorded by the manufacturer as 0.3 g per 100 cc.

TABLE I.

Plasma Sodium Concentration (Milli-equivalents per liter) Before and at Various Intervals Following the Injection of Salt poor Human Albumin and Double Strength Plasma in Patients with Malnutrition.

Subject	Age	Sex	Diagnosis	Injection	Plasma Na^+ concentration (meq.)						
					Before	After	1 hr	3 hr	6 hr	24 hr	Max. decrease (meq)
B.E.	71	M	Carcinoma head of pancreas	Albumin (50 g)	150	138	150	156	147		— 12
				" "	150	149	148	149	142		— 8
G.H.	45	F	Non-tropical sprue	" "	137	123				144	—14
				" "	144	130				131	—14
B.P.	48	M	Cirrhosis	" "	136	137	137	141		145	—
				" "	143	130	146			147	— 13
E.R.	75	M	Carcinoma of pancreas	" "	149	139.5	139.5	141.5	143.5	144	— 9.5
				Plasma (470 cc double strength)	141	140	141	148	139	141	—
G.S.	71	M	Cirrhosis	Plasma (285 cc double strength)	138	142	141	148		138	

Discussion. The fall we observed in the plasma sodium concentration following either hemorrhage or the injection of 25% salt-poor human albumin solution is assumed to be a part of the hemodilution process and thus resolves itself ultimately to a discussion of the source of the fluid that enters the circulation following such procedures.

In a review of the literature we have found but one report on the changes in serum sodium and potassium following hemorrhage. Stewart and Rourke[6] reported that in 4 normal dogs bled 2 to 3.5% of their body weight, samples drawn 12 hours after the hemorrhage showed a fall in serum sodium, potassium and protein concentration, and variable concentrations of the chloride, carbon dioxide and non-protein nitrogen. These changes occurred whether the animals were allowed fluids by mouth or not. Our subjects were given neither food nor fluids by mouth during the first hour. They remained in bed until the ½ hour samples had been drawn and were then permitted to be up and about. After the one-hour sample was drawn food and fluids were permitted *ad libitum.* The data presented herein in normal human subjects confirm Stewart and Rourke's findings in dogs.

Ashworth, Muirhead and Hill[4] studied the effect of concentrated citrated and defibrinated dog and human plasma on the serum sodium and potassium concentration of normal dogs. They found no change in the samples drawn 15, 20, 60 minutes and 4 hours after injection. Since the plasma volume (dye method) as well as the extracellular fluids (thiocyanate method) increased after infusion, without change in serum sodium concentration, these authors concluded that the fluid added to the circulation was derived from the intracellular space. We observed an increase in the serum sodium concentration 3 hours after the injection of concentrated citrated plasma in 2 patients.

Heyl, Gibson and Janeway[5] in their study of the effect of concentrated solutions of

[4] Ashworth, C. T., Muirhead, E. E., and Hill, J. M., *Am. J. Physiol.*, 1942, **136**, 194.

[5] Heyl, J. T., Gibson, J. G., 2nd, and Janeway, C. A., *J. Clin. Invest.*, 1943, **22**, 713.

human and bovine serum albumin on the blood volume after acute blood loss in normal men, reported no change in the serum or urinary potassium concentration, but their albumin solutions were not salt-poor. In 5 of the 7 patients in whom we injected salt-poor albumin a significant fall in plasma sodium occurred. In the single patient in whom potassium was measured, no change was noted. It should be emphasized that the fall in sodium concentration was most marked immediately after infusion, at which time the plasma volume had increased by a volume more than 3 times that of the fluid injected. The magnitude of the fall in sodium concentration was much less than could be accounted for by hemodilution with a sodium-free solution, hence the fluids responsible for the dilution probably contained sodium but in lower concentration than in plasma.

Because intracellular fluid is poor in sodium and rich in potassium, it is logical to assume that much of the diluting fluid must have come from this source, in which case one might expect a rise in plasma potassium. The latter was measured in 2 cases, but no change was found. However, renal clearance of potassium is so rapid that this discrepancy may not be significant. Indeed, Stewart and Rourke,[6] in the experiments cited earlier, found an increase in potassium output in the urine after hemorrhage. The red cells themselves might be assumed to be the source of the diluting fluid, but this could be true only if a rapid two-way shift of fluid were involved, inasmuch as no change in the size of red cells was observed after the injection of albumin.[2]

Summary. 1. Following phlebotomy in each of 4 normal male subjects, there was a transient fall in the plasma sodium concentration.

2. Following injections in 4 patients of a 25% solution of salt-poor human albumin solution, a similar fall in the plasma sodium concentration was noted.

3. It is concluded that sodium-poor fluid is added to the plasma, presumably from the intracellular space, following hemorrhage, as well as following injection of salt-poor human albumin.

[6] Stewart, J. D., and Rourke, G. M., *J. Clin. Invest.*, 1936, **15**, 697.

16360

Environmental Conditions Which Initiate Sweating in Resting Man.

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During the course of studies[1,2,3] on the influence of environmental conditions upon the rate of water loss from the skin of man, observations were made to learn the environmental temperature and relative humidity which would initiate sweating in normal man.

Eleven subjects of both sexes, varying in ages from 12 to 59 years, were permitted to rest quietly on a hospital type of bed in an air-conditioned room. They were clothed only in a cotton gown. The rate of water loss from the skin of the epigastrium and volar surface of the right forearm was determined by a method previously described.[4]

* Aided by grants from the Life Insurance Medical Research Fund, a War Contract No. WD-49-007-MD-389, Helis Institute for Medical Research, and the Mrs. E. J. Caire Fund for Research in Heart Disease.

[1] Burch, George, and Winsor, Travis, *J. Clin. Invest.*, 1944, **23**, 937.

[2] Winsor, Travis, and Burch, George E., *Arch. Int. Med.*, 1944, **74**, 428.

[3] Burch, George E., and Winsor, Travis, *Arch. Int. Med.*, 1944, **74**, 437.

[4] Burch, George E., and Sodeman, William A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 190.

TABLE I. Results of Studies to Learn Environmental Conditions Necessary to Initiate Sweating in Normal Man Resting in a Hospital Type Bed.*

Subject	Age	Sex	Color	Before sweating				Sweating			
				Temp. (C)	Relative humidity (%)	Rate of water loss mg/cm²/10 min.		Temp. (C)	Relative humidity (%)	Rate of water loss mg/cm²/10 min.	
						Epigastrium	Forearm			Epigastrium	Forearm
1	22	F	C	35	85	6.0	4.2	37.8	60	19.8	8.9
2	50	F	W	32.2	54	7.1	6.8	34.4	50	8.7	9.5
3	25	F	W	34.4	28	7.4	4.5	36.7	26	13.3	6.3
4	40	M	W	30.0	62	5.8	6.1	33.8	58	7.3	7.0
5	12	F	C	32.2	90	4.5	7.5	34.4	70	7.5	5.9
6	31	F	C	32.2	80	5.6	5.4	35.5	56	11.6	4.5
7	49	F	C	31.1	42	5.2	5.5	33.3	42	20.0	18.1
8	34	M	C	36.1	26	4.6	3.6	38.9	28	7.5	4.1
9	19	M	C	38.9	9.5	5.5	4.1	38.9	9	6.9	4.3
10	17	M	W	33.3	22	7.0	2.8	35.5	20	11.4	11.0
11	59	M	W	33.3	26	4.0	4.2	34.4	26	5.9	6.0
Mean				33.5	47.9	5.7	5.0	35.8	40.5	10.9	7.8
Max.				38.9	90	7.4	7.5	38.9	70	20.0	18.1
Min.				30.0	9.5	4.0	2.8	33.3	9	5.9	4.1

* The water lost before sweating is lost by diffusion. The onset of sweating in subjects Nos. 5 and 6 showed local variations.

After the subjects had rested for at least 60 minutes under comfortable environmental conditions (temperature and relative humidity approximately 20.4°C and 50% respectively) the room temperature was elevated 3°C at a time, and the relative humidity was changed in a variable fashion. After the subjects had been exposed for 15 minutes to the new room conditions their rate of water loss was measured. This procedure was continued until an atmospheric condition was reached which was associated with a definite increase in rate of water loss. Occasionally, as a check, the room temperature or humidity or both were lowered, the rate of water loss was measured, the temperature or humidity or both were raised again, and another water collection was made.

Results are summarized in Table I. Sweating was initiated by a temperature of about 36°C when the relative humidity was about 40%; with a low relative humidity, a higher air temperature was required. Sweating began at a temperature of 39°C, even though the relative humidity was as low as 9%. In one patient sweating was cyclic, *i.e.*, it alternately occurred and ceased for 15-minute periods, even though the room conditions remained unchanged. These observations suggested that sweating cooled the subject sufficiently to abolish the need for this mechanism for a certain length of time. He accumulated heat, sweated again, cooled his body, and then stopped sweating. This cyclic sweating conserves water and electrolytes.

Such observations indicate that environmental temperature of 34.4°C and relative humidity of about 50% are essentially the threshold level for sweating in normal man resting in bed. Increased exercise and rate of heat production are accompanied by a proportionate lowering of the threshold.

When the studies were prolonged, subjects became restless and irritable, and "nervous" or "psychogenic" sweating resulted, which made it impossible to observe the thermal sweating.

Summary. Observations were made on 11 subjects of varying ages to discover the environmental conditions necessary to initiate

sweating in man. These studies, performed in a room designed for close control of temperature and humidity, indicate that an environmental temperature of 34.4°C and relative humidity of about 50% are essentially the threshold level for sweating in normal man resting in bed. Exercise and increased rate of heat production are associated with a proportionate lowering of the threshold.

16361

Utilization of Glutamic Acid in the Presence of High Levels of Pteroylglutamic Acid.

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It has been recently suggested that pteroylglutamic acid (PGA) may function as a metabolic antagonist for glutamic acid.[1,2] The statement was made that "folic acid may competitively interfere with the nutrition of the spinal cord just as certain vitamin deficiencies in experimental animals may be caused by closely related chemicals. Thiamine deficiency, for example, may be induced by the administration of pyrithiamine, and pantothenic acid deficiency by pantoyltaurine." It was further suggested that PGA could interfere with the metabolism of the central nervous system *in vivo* or *in vitro*.[1]

The analogy between folic acid and such "anti-vitamin" compounds as pyrithiamine appears to be inappropriate. Folic acid is a member of the vitamin B complex, and is itself inhibited by "anti-vitamin" compounds which are comparable to pyrithiamine and pantoyltaurine. These compounds include "methyl folic acid,"[3,4] pteroylaspartic acid,[5] 4-amino pteroylglutamic acid,[6] certain pteridines,[7] a sulfonyl-substituted benzimidazole analogue of PGA,[8] and N^{10}-methyl pteroic acid.[9]

Since representative species of bacteria require both folic acid and glutamic acid, it is obvious that folic acid does not competitively interfere with the metabolism of glutamic acid by these bacteria. However, because the response to glutamic acid obtained with *L. casei* and *S. faecalis* R is so quantitative and sensitive, an experiment was made to determine whether massive amounts of PGA would slow up growth on suboptimal levels of glutamic acid. A culture medium deficient in glutamic acid[10] was used and PGA was added at levels of 0.01 μg, 1 μg, 10 μg, to 3 series of tubes containing varying amounts of glutamic acid. The results are illustrated in Fig. 1.

The growth curves indicate that increasing the level of PGA 1000-fold had no inhibitory

1 Anonymous, *New England J. Med.*, Editorial, 1947, **237**, 713.

2 Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, **3**, 68.

3 Martin, G. J., Tolman, L., and Moss, J., *Arch. Biochem.*, 1947, **12**, 318.

4 Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427; Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 368.

5 Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, 1947, **170**, 323.

6 Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

7 Daniel, L. J., Norris, L. C., Scott, M. L., and Heuser, G. F., *J. Biol. Chem.*, 1947, **169**, 689.

8 Edwards, P. C., Starling, D., Mattocks, A. M., and Skipper, H. E., *Science*, 1948, **107**, 119.

9 Smith, J. M., Jr., and Cosulich, D. B., *J. Am. Chem. Soc.*, in press.

10 Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C., *J. Biol. Chem.*, 1944, **155**, 591.

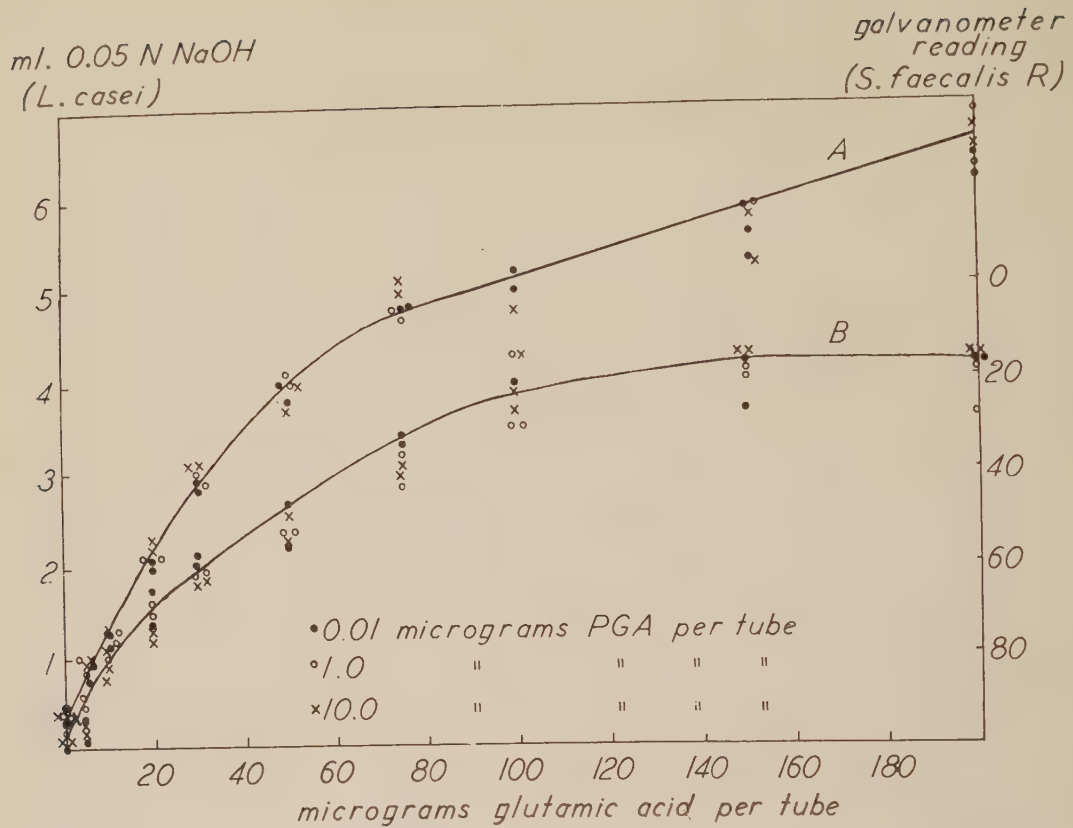

FIG. 1.

Growth response of *Lactobacillus casei* (curve A) and *Streptococcus faecalis* R (curve B) at 72 hours to glutamic acid in the presence of various levels of pteroylglutamic acid (PGA).

effect upon the utilization of glutamic acid by either *L. casei* or *S. faecalis* R. The scattering around the experimental points was within the anticipated limits of the assay method and in no case was the trend dependent upon the level of PGA. The experiment also demonstrates that neither organism is able to use PGA as a substitute for glutamic acid.

In view of the suggestion that PGA interferes with the metabolism of glutamic acid by the central nervous system *in vitro,* further experimental work was done to determine the effect of PGA on the utilization of glutamic acid by brain and kidney slices. The slices were suspended in Ringer-phosphate solution and the rate of oxygen consumption was determined at 37° in the Warburg-Barcroft apparatus. The minimum concentration of glutamic acid (3×10^{-3}M) which caused an appreciable stimulation of oxygen consumption, was used, for this concentration would presumably give the most favorable conditions for observing any competitive interference with the utilization of this acid.

The results are given in Table I. Q_{O_2} values (cu mm of oxygen consumed per mg dry tissue per hour) are included for brain slices for both the first hour and the first 3 hours of respiration, for the consumption of oxygen was not a linear function with time. The results can be evaluated by examining the values obtained for increases in Q_{O_2} caused by adding glutamic acid to the medium as summarized in the last 2 columns of Table I. When the concentrations of glutamic acid and PGA were equimolar there was no significant depression in the utilization of glutamic acid by brain or kidney slices. Had the PGA interfered with the utilization of glutamic acid, the addition of PGA should have

TABLE I.
Effect of Addition of Pteroylglutamic Acid to the Medium on Utilization of Glutamic Acid by Surviving Brain and Kidney Tissue Slices of Normal Rats.

		Q_{O_2}									Increase in Q_{O_2} with addition of glutamic acid*	
		Brain slices						Kidney slices				
		1st hr			3-hr interval							
Exp.	PGA conc.	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	Brain	Kidney
1	0	3.4	4.6	6.1	1.9	3.4	4.7	10.4	12.8	13.3	2.8	2.9
	3×10^{-4} M	3.4	5.8	6.3	1.9	4.2	5.0	9.2	11.8	12.0	3.1	2.8
2	0	5.0	6.8	9.6	3.3	5.2	7.4	11.8	19.2	21.9	4.1	
	3×10^{-4} M	4.4	8.2	9.8	3.0	6.1	7.6	11.6	19.6	—	4.6	
3	0	4.6	6.0	7.2	2.8	4.3	5.4	9.9	12.0	13.1	2.6	3.2
	3×10^{-3} M	3.6	6.0	6.8	2.2	4.2	5.2	9.0	11.6	12.7	3.0	3.7
4	0	5.0	8.6	7.3	3.3	6.4	6.0	15.0	15.2	18.0	2.7	3.0
	3×10^{-3} M	4.4	7.2	7.9	2.9	5.3	6.0	12.2	15.2	14.1	3.1	1.9

(a) No glutamic acid added to medium.
(b) Medium contained 3×10^{-3} M added glutamic acid.
(c) Medium contained 10^{-2} M added glutamic acid.
*The values are calculated from the results with 10^{-2} M glutamic acid. The values for brain slices were determined from the 3-hour respiration interval.

reduced the magnitude of the Q_{O_2} increases, but no such reduction was observed. The maximum PGA level which has been reported in the blood of human subjects is 0.8 μg per ml[11] following the intravenous administration of 15 mg of PGA. In the present study levels as high as 1323 μg PGA per ml of medium were without significant effect on glutamic acid metabolism.

In another series of similar experiments, the animals were either made severely deficient in PGA or fed a high level of PGA for a prolonged period. The birds on the deficient diet developed a severe cervical paralysis,[12,13] and the deficient rats showed a mild cytopenia. The animals were sacrificed when the marked signs of the characteristic deficiency developed. Control animals were fed purified diets[4,12] containing adequate levels of PGA (1 mg PGA per kg diet for rats and 2 mg per kg of diet for turkeys). Because of the non-linear oxygen consumption curves obtained with brain tissue the control animals were sacrificed simultaneously with the experimental animals and the tissue slices were prepared from both at the same time. The respiration of the tissue slices was measured as described above, except that no PGA was added to the medium. No difference between the behavior of the tissues from the deficient and control animals was found (Table II).

The effects of feeding large amounts of PGA and of PGA deficiency in the rat on glutamic acid metabolism by brain and kidney tissue *in vitro* were compared (Table II). The "high-PGA" diets contained 100 mg PGA per kg and were fed for several weeks. The tissues from two series of animals showed no significant differences in the tests.

Discussion. In studies with various species of animals PGA, like the other B-complex vitamins, has been shown to be a substance of very low toxicity.[14] Prolonged dosage of human subjects with 50 mg of PGA daily has been reported not to produce adverse symptomatology.[15] Recent statements[2] have

11 Denko, C. W., Abstracts of Papers, American Chemical Society, 112th meeting, New York, 25 C, 1947.

12 Jukes, T. H., Stokstad, E. L. R., and Belt, M., *J. Nutrition*, 1947, **33**, 1.

13 Richardson, L. R., Hogan, A. G., and Kempster, H. L., *J. Nutrition*, 1945, **30**, 151.

14 Harned, B. K., Cunningham, R. D., Smith, H. D., and Clark, M. C., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 289.

15 Berry, L. J., and Spies, T. D., *Blood*, 1946, **1**, 271.

TABLE II. Effect of Glutamic Acid on Respiration Rate of Surviving Brain and Kidney Slices from Animals Which Had Received Diets Containing Varying Levels of PGA.

Exp.	Animal	Wt, g	PGA dietary supplement, mg/kg	Time on diet, days	Q_{O_2} Brain Slices 1st hr (a)	1st hr (b)	1st hr (c)	3-hr interval (a)	3-hr interval (b)	3-hr interval (c)	Kidney slices (a)	Kidney slices (b)	Kidney slices (c)	Increase in Q_{O_2}, with addition of glutamic acid* Brain	Kidney
1	Turkeys	190	0	21	6.5	8.6	9.8	4.9	7.0	7.9				3.0	
		165	2	21	6.5	9.1	9.8	4.7	7.6	7.9				3.2	
2	,,	210	0	24	7.9	10.4	10.8	5.7	7.7	9.0				3.3	
		220	2	24	8.9	9.8	9.8	6.2	7.8	8.3				2.1	
3	Rat	110	0	42	3.5	7.7	8.5	2.3	5.8	6.3	14.0	17.2	17.6	4.0	3.6
		130	1	42	4.6	9.9	10.0	3.0	7.0	7.3	14.0	16.8	21.2	4.3	7.2
4	,,	120	0	44	6.4	8.4	9.2	4.0	6.4	6.9	17.4	18.0	22.0	2.9	4.6
		182	1	44	6.6	8.3	9.7	4.3	6.3	7.3	13.6	16.8	21.6	3.0	8.0
5	,,	230	100	23	3.7	5.7	8.6	2.3	5.1	6.9	10.0	15.7	17.8	4.6	7.8
		270	1	23	4.6	6.6	8.2	2.8	4.7	6.6	11.5	15.2	18.2	3.8	6.7
6	,,	270	100	27	5.3	6.9	6.7	3.5	5.4	5.7	10.3	14.2	18.6	2.2	8.3
		210	1	27	4.1	6.4	7.2	2.7	4.8	5.5	10.3	16.7	18.6	2.8	8.3

(a) No glutamic acid added to medium. (b) Medium contained 3×10^{-3} M added glutamic acid. (c) Medium contained 10^{-2} M added glutamic acid. * See footnote in Table I.

implied that glutamic acid is important for the functioning of the choline acetylase system in the brain and that PGA might competitively interfere with glutamic acid in nerve metabolism. Although earlier studies[16] related glutamic acid to the choline acetylase system, reactivation of this system by glutamic acid has been since shown to be a non-specific effect, also exerted by various other amino and organic acids, especially citric acid.[17] In fact, recent purification of the system has led to the conclusion that acetate rather than citrate[18] is responsible for the effect. That PGA plays a positive role in the functioning of the central nervous system has been shown by the development in young turkeys of generalized paralysis on PGA-deficient diets[12,13] and that the paralysis disappears within a few hours after the injection of PGA.[13] The present investigation indicates that the respiration *in vitro* of brain tissue slices in turkeys with this paralysis, was not grossly different from that of brain tissue slices from control birds.

Summary. The utilization of glutamic acid by *Lactobacillus casei* and *Streptococcus faecalis* R was not affected by increasing the pteroylglutamic acid (PGA) content of the culture medium a thousandfold. The respiration of rat brain slices was not inhibited by adding PGA to the medium at a level of 3×10^{-4}M, more than 100 times the highest concentration observed in the blood of human subjects following the intravenous injection of 15 mg of PGA. The increased respiration of rat brain slices produced by adding glutamic acid was not reduced by adding PGA to the medium at concentrations of 3×10^{-3}M and 3×10^{-4}M. The respiration rate of brain slices of PGA-deficient turkeys, with or without added glutamic acid, was not consistently different from that of brain slices from control birds which had received an adequate

[16] Nachmansohn, D., and John, H. M., *J. Biol. Chem.*, 1945, **158**, 157.

[17] Feldberg, W., and Mann, T., *J. Physiol.*, 1946, **104**, 411; Lipton, M. A., and Barron, E. S. G., *J. Biol. Chem.*, 1946, **166**, 367.

[18] Kaplan, N. O., and Lipmann, F., *Fed. Proc.*, 1947, **6**, 266.

dietary supplement of PGA. Brain and kidney tissue slices from rats receiving a high dietary level of PGA respired at the same rate as corresponding tissues from control rats, with or without added glutamic acid.

ADDENDUM: After this manuscript was submitted, an article appeared (Grossowicz, N., *J. Biol. Chem.*, 1948, **173**, 729) which reported that glutamine inhibited the growth-promoting effect of glutamic acid for *Staphylococcus aureus*, and and that this inhibitory effect of glutamine could be overcome by pteroylglutamic acid, by glutathione, or by extra glutamic acid. These results apparently indicate that under certain circumstances pteroylglutamic acid and glutamic acid may function interchangeably in the nutrition of micro-organisms.

16362 P

Effect of Tween 80 on Certain Strains of *C. diphtheriae*.

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In attempts to prepare a fluid synthetic medium for general use in diphtheriology, difficulty was encountered in that recently discovered minimus strains[1,2,3] and a few, closely related, minute-colony strains, failed to grow in a medium suitable for most other strains. The synthetic medium used consists of amino acids, inorganic salts, carbohydrates and growth factors in as pure form as could be obtained. It has no peptone, casein or other constituents of variable or unknown composition. Details of the medium will be published elsewhere.

The minimus strains have also uniformly failed to grow well in infusion broth. The granular nature of growth of the minimus strains in infusion broth suggested the possibility that a surface tension reducent might facilitate growth, following the line of thought suggested by Dubos *et al.*[4,5,6] in their studies of the tubercle bacillus. Accordingly, Tween 80 was added aseptically from a freshly prepared, sterile, aqueous solution, in a concentration of about 0.05%, to the synthetic medium just before inoculation. Immediate and striking stimulation of growth of the minimus-type strains was observed. Concentrations of Tween up to about 0.5% have since been used with no apparent inhibition of the minimus type. No optimum or inhibitory range has yet been determined.

With other types of diphtheria bacilli, the results of adding Tween 80 to the medium are somewhat variable. Some strains, particularly of the mitis type, appear slightly inhibited; others show little or no difference in growth response to Tween. Gravis and gravis-like strains which normally grow with pellicle formation, lose their pellicle and grow diffusely throughout the medium. No types other than minimus are stimulated to any great degree, with the exception of a few minute-colony forms sent by Dr. McLeod from Leeds sometime ago. These strains were designated by McLeod as the intermedius type. It is of interest that the 4 strains of this group which Frobisher found to be identical with minimus type[3] were more markedly stimulated by addition of Tween than were the other 6.

Similar stimulation of growth of the mini-

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1 Eller, C. H., and Frobisher, M., Jr., *Am. J. Hygiene*, 1945, **42**, 179.

2 Frobisher, M., Jr., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 330.

3 Frobisher, M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 304.

4 Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

5 Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 56.

6 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

mus-type strains was observed when Tween 80 was added to tubes of heart-infusion broth. The difference in growth resulting from addition of Tween 80 to this medium was not so striking as in the synthetic medium, since growth of the minimus strains is normally better in heart-infusion than in synthetic medium. Again, the results with strains of other types were variable. None, other than minimus type, was definitely stimulated, with the exception of the minute-colony forms noted above.

Plates of McLeod's chocolate agar were prepared; half with about 0.3% Tween, and half without. A small loopful of 24-hour broth culture of various types of diphtheria organisms was streaked on both media. Results of this experiment paralleled those obtained with liquid media. Minimus colonies were markedly increased in size, from less than 0.1 mm diameter on plates without Tween to diameters of 2.0 to 3.0 mm on the plates with Tween added. Colony size of other types was variable, the intermedius strains of McLeod being the only ones which showed any significant increase in diameter (0.1 mm to 1.5 mm).

It has been repeatedly observed in this laboratory[7] that at least 1 ml of 48-hour culture of the minimus type organisms is required to kill guinea pigs and that even that dose kills irregularly; whereas, the lethal dose of the gravis and mitis types approximates 0.2 ml or less. Though the minimus strains grow more sparsely than do the other types, after seeing the broth cultures it is a little difficult to accept the idea that this difference in lethal dose is due to *numbers* of organisms alone. Therefore, 48-hour broth cultures of a minimus strain, grown with 0.05% Tween, and in the same medium without Tween, were injected intradermally into duplicate guinea pigs in amounts of 0.2, 0.5, and 1.0 ml. All animals receiving the Tween culture were dead within 72 hours. Autopsy showed typical signs of diphtheritic deaths. Only one pig with the culture without Tween died, and that was one of the two which had received 1.0 ml of culture. All other experimental and control animals survived without evidence of disease for more than two weeks. This experiment had been repeated in part with a second minimus strain, with the same results.

In alcohol-ether and acetone fractionation of the gravis, mitis and minimus types, Parsons[8] found that the minimus strains contained more lipid material than did gravis or mitis strains. This suggests a relationship of wetting agent (Tween 80) to the lipid-rich minimus type, analogous to the relationship of the same wetting agent to the lipid-rich tubercle bacilli, shown to exist by Dubos *et al.* The surface tension of the synthetic medium was found to be relatively high. Tween 80 reduces this surface tension markedly. That the surface tension of the heart-infusion without Tween is considerably lower than that of the synthetic medium might partially account for the normally better growth of the minimus strains in heart-infusion broth.

The role of Tween as a non-toxic source of oleic acid must also be considered in view of the work of Cohen *et al.*,[9] who found oleic acid to be one of two growth factors, present in serum and milk, essential for a gravis strain when very minute inocula were used on solid medium. No statement was made by these authors as to the effect of these factors in liquid media.

Summary. It has been observed that the addition of Tween 80 to solid and liquid media markedly stimulates the growth of minimus type diphtheria bacilli. This suggests a relationship between this wetting agent and the lipid-rich minimus organisms analogous to the relationship of the same agent to the lipid-rich tubercle bacilli, demonstrated by Dubos *et al.* There is also evidence that Tween 80 enhances the virulence of the minimus strains.

The writer is indebted to Dr. Martin Frobisher, Jr., for his stimulating interest and help and Dr. Elizabeth I. Parsons for her permission to cite unpublished data.

7 Frobisher, M., Jr., and Parsons, E. I., unpublished data.

8 Parsons, E. I., unpublished data.

9 Cohen, S., Snyder, J. C., and Mueller, J. H., *J. Bact.*, 1941, **41**, 581.

16363

Hyaluronidase Content of Normal and Inflamed Guinea Pig Skin.

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In the presence of hyaluronidase the epidermal challenge reaction in guinea pigs sensitized to paraphenylenediamine is markedly increased as compared to control animals (Mayer and Kull[1]). This observation suggested that hyaluronidase plays a role not only in the spread of invasive organisms and the ensuing bacterial inflammation (Duran-Reynals[2]), but also in certain non-bacterial inflammations of the skin.

In order to obtain further evidence of this hypothesis it was desirable to determine whether the hyaluronidase content of the guinea pig skin was increased during allergic and non-allergic inflammations.

Methods. Guinea pigs were sensitized to paraphenylenediamine in the manner described by one of us.[3] After sensitization had been established, the animals were challenged with 10% paraphenylenediamine in petrolatum applied to the left flank, and sacrificed 24 hours later. 3 x 4 inch strips of skin were removed from challenged and unchallenged sites.

Physical inflammations were produced by application of heat (water at 70°C) for 1 and 2 minutes respectively on the shaven skin and by ultraviolet irradiation (Hanovia lamp, distance 12 inches) for various exposure periods. Skin samples were obtained from both inflamed and normal sites.

Identical weights (based on dry weight) of inflamed and non-inflamed skin were successively subjected to freezing and thawing and then ground with sterile sand. The resulting tissue-brei was extracted with 10 ml of 0.1 M acetate buffer, pH 6.0, containing 0.15 M NaCl, and centrifuged. The supernatant fluid was assayed viscosimetrically according to the method of McClean and Hale,[4]

TABLE I.

Treatment	Guinea pig No.	Intensity of inflammation	Hyaluronidase content skin-viscosity reduction units per g dry wt*	
			Normal skin	Inflamed skin
Paraphenylene diamine dermatitis	1	++++	.015	.456
	2	++++	.043	.185
	3	++++	.157	.925
	4	++++	.022	.856
	5	++++	.045	1.110
Heat-induced inflammation	6	++++	.00	.391
	7	+++++	.034	.556
Ultraviolet erythema	8 (2′†)	+	.040	.0061
	9 (5′)	+	.00	.054
	10 (20′)	++	.0126	.034
	11 (30′)	++	.045	.0

* The skin viscosity reduction unit was obtained by applying the formula of McClean and Hale[4] with the modification of 60′ being equivalent to one unit. The values are extrapolated by applying $\sqrt{}$ time vs. viscosity.

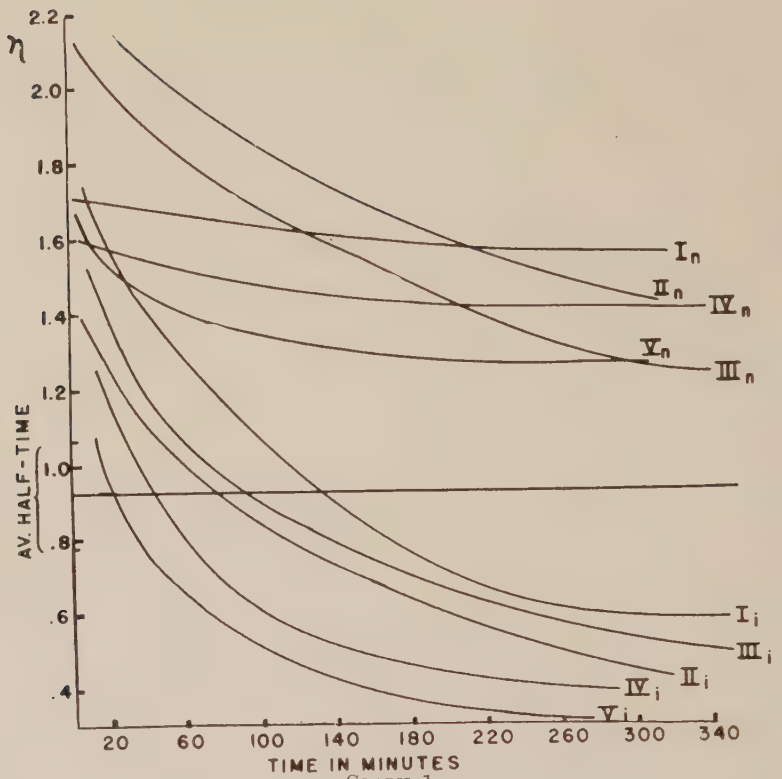
† Minutes of exposure to ultraviolet. One minute equal to one guinea pig skin erythema dose.

1 Mayer, R. L., and Kull, F. C., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 392.

2 Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

3 Mayer, R. L., *Arch. f. Dermat. u. Syph.*, 1931, **163**, 223.

4 McClean, D., and Hale, C. W., *Biochem. J.*, 1941, **35**, 159.



GRAPH 1.

Viscosity Reduction with Normal and Inflamed Skin from Guinea Pigs Allergic to Paraphenylenediamine.

In, IIn, IIIn, IVn, and Vn are the viscosity reduction curves obtained from normal skin; Ii, IIi, IIIi, IVi, and Vi are the viscosity reduction curves obtained from inflamed skin.

using 0.4 cc of the above supernate and 0.6 ml of 0,417% hyaluronate (prepared from umbilical cords according to Kass and Seastone[5]) dissolved in the acetate buffer.

Results. The results of the experiments on 11 animals shown in Table I indicate that the free hyaluronidase content in allergic or heat-inflamed skin is up to 40 times as high as that of the corresponding normal skin.

Unlike the inflammations induced by the allergic challenge or by heat, the 24-hour ultraviolet reaction after the administration of 2 to 60 guinea pig erythema doses did not result in a consistent increase of free hyaluronidase. No increases were observed in 2 skins which had been irradiated for 2- and 30-minute periods respectively, while 2 other skins, irradiated for 5- and 20-minute periods respectively showed a slight but not significant increase.

Negative results were also obtained with 2 guinea pigs sensitized to horse serum upon subjection to local Arthus reactions.

Graph 1 shows the course of viscosity reduction of the tests in animals presenting allergic inflammation (animals 1-5).

Discussion. While hyaluronidase in bound form is known to exist in the normal skin (Meyer[6]), our experiments have shown that normal guinea pig skin also contains very small amounts of free hyaluronidase. During inflammation produced by a challenging application of paraphenylenediamine on sensitized skin or after treatment of the skin with hot water, the amounts of polysaccharide-depolymerizing enzymes (probably chiefly hyaluronidase) are markedly increased—up

[5] Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

[6] Meyer, K. F., *Physiol. Rev.*, 1947, **27**, 335.

to 40 times.

Ultraviolet irradiation, producing definite erythema in guinea pigs, results in an occasional but very slight increase of these enzymes, contrary to the Arthus phenomenon, which in 2 rabbits did not result in increased amounts. It is noteworthy that the ultraviolet treatment and the Arthus phenomenon primarily concern the cutis, whereas the heat-induced inflammation and the epidermal allergic challenge reaction concern mainly the epidermal tissue.

Although the previous experiments on the influence of antihistaminics upon the hyaluronidase reaction[1] suggest a relationship between skin inflammation and the liberation of hyaluronidase, further experiments are necessary to determine whether hyaluronidase plays an active role during these inflammations or is only a concomitant factor subsequent to an inflammatory process. Another unsolved problem is the source of the liberated enzyme.

Conclusion. The content of polysaccharide-depolymerizing substances (probably chiefly hyaluronidase) during allergic dermatitis (paraphenylenediamine) and heat-induced inflammation on guinea pig skin is markedly increased.

16364

Effect of Intravenous Cytochrome C on Capacity for Effort Without Pain in Angina of Effort.

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Within the past few years, reports have appeared on the effect of Cytochrome C* on tissue anoxia. From the clinical point of view, Proger and his co-workers have presented evidence that myocardial anoxia can be relieved by intravenous injection of Cytochrome C, as demonstrated by the prevention or reversal of the electrocardiographic changes induced by breathing 10% oxygen.[1] It is their contention that, under such circumstances, Cytochrome C enhances the uptake of oxygen by the myocardium.

Another method of testing the effect of a drug on myocardial anoxia is to determine the capacity for effort without pain in patients who have angina pectoris brought on by effort. Since this method depends on a subjective end-point, it is necessary to use careful controls and a completely "blind" technique, as described by Gold and his co-workers, in order to eliminate variable psychosomatic effects.[2] This method has been used by us in an evaluation of the effect of intravenous aminophylline on angina of effort, and it proved to be a satisfactory investigative technique.[3]

The subjects of the study were all selected from the active attendance of the Cardiac Clinic. All of them had either arteriosclerotic or hypertensive heart disease or a combination of both, and all presented the symptom of chest pain on effort. No patients were included in this study who had spontaneous chest pain unrelated to effort, excitement, heavy meals, or very cold weather. Also excluded were patients who showed any clinical evidence of congestive failure.

The method consisted of determining the subject's capacity for effort without pain by having him walk back and forth over a

* Cytochrome C used in this study was furnished by Wyeth, Inc., Philadelphia, Pa.

1 Proger, S., and Dekaneas, D., *J. Pediat.*, 1946, **29**, 729.

2 Gold, H., Kwit, N. T., and Otto, H., *J. A. M. A.*, 1937, **108**, 2175.

3 Bakst, H., Kissin, M., Leibowitz, S., and Rinzler, S., *Am. Heart J.*, in press.

standard set of steps until the onset of his usual type of chest pain. The rate of walking was set by the subject himself, and in any subsequent tests on that same subject, he was kept to the same rate. Every test with Cytochrome C was coupled with a control test on the same day, thus eliminating the effect of the spontaneous variations in pain on different days in the same subject. All tests were preceded by a rest period of one hour, and were conducted in the early afternoon, at least 4 hours after the last meal. In a typical experiment, the patient came to the laboratory at 12:30 o'clock without having had any lunch. He rested at least one hour and was then given the first intravenous injection at a rate of one cc per minute (total of 5 cc per injection). This was followed by a 5 minute rest period, and then the first trial was carried out. As soon as the end-point was reached, the subject sat down and rested for another hour. He was then given the second injection in the same manner, followed by a 5 minute interval, and then the second trial was conducted.

Cytochrome C was given intravenously in doses of 50 mg in 5 cc of solution. The control material used was physiological saline solution, and because Cytochrome C is red, it was necessary to prevent the patient from seeing what was being injected, lest he have a different psychological effect from a red medicine than from a white one. For this reason, a cloth screen was interposed between the patient and the investigator giving the injections, and the subject's arm was extended through a slit in the screen. The injections were all given by one person, and the observations on capacity for effort were made by the other investigator, who was unaware of which substance had been administered. The sequence of injections was varied so that in some instances the control test preceded the test with Cytochrome C, and in other instances the order was reversed. There was no indication that the subjects of the study were aware that different materials were being injected. There were no consistent reactions to the injection of Cytochrome C which would enable the subjects to detect that they were receiving a material different from the control solution.

Results. There were 12 pairs of tests conducted on 8 patients. In 4 of the pairs, the trial with Cytochrome C preceded the control trial; in the remaining 8 pairs, the control trial was conducted first. Analysis of the results reveals that Cytochrome C produced no increase in capacity for effort without pain. In fact, there was a mean decrease in performance equal to 2.33 $\pm$ S.E. 1.95 trips following the injection of Cytochrome C, as compared with the results obtained with the control injections. Expressed in percentages, this is a decrease of 8.7 $\pm$ 7.2%. Since the "t" value derived from these figures is only 1.19, it is concluded that there is no statistically significant difference between the results obtained following injection of Cytochrome C and the results following injection of physiological saline. A summary of the results is found in Table I.

Discussion. Cardiac pain as exemplified by angina of effort has been suggested to be due to an inadequate supply of oxygen to the heart to meet its demands.[4] The stimulus for pain is believed to be an accumulation of acid metabolites produced by the heart muscle under ischemic conditions.[5] The possibility of enhancing the tissue uptake of oxygen by means of Cytochrome C in instances of myocardial anoxia is then of special interest in patients with angina of effort. Proger and Dekaneas showed that 50 or 60 mg of Cytochrome C injected intravenously was able to revert to normal in as little time as two minutes an abnormal electrocardiogram produced by breathing a 10% oxygen mixture.[1] Rabinowitch, Elliott, and McEachern have shown that Cytochrome C in doses of 50 mg is taken up by tissues or destroyed within a matter of minutes after injection.[6]

We can assume that the dose of 50 mg of Cytochrome C given intravenously is taken

[4] Keefer, C. S., and Resnik, W. H., *Arch. Int. Med.*, 1928, **41**, 469.

[5] Katz, L. N., *Am. Heart J.*, 1935, **1**, 322.

[6] Rabinowitch, R., Elliot, K. A. C., and McEachern, D., *Canad. M. A. J.*, 1948, **58**, 92.

TABLE I.
Clinical Data and Results of Exercise Tolerance Tests.

Subject	Sex	Age	Diagnosis	No. of trips performed 1st trial	2nd trial
A.L.	M	55	Arteriosclerosis, hypertension, enlarged heart, dilated aorta. ECG: Prolonged A-V conduction	24 (S)*	27 (C)†
J.G.	M	63	Arteriosclerosis, enlarged heart, coronary sclerosis. ECG: Ventricular premature contractions	12 (S)	11 (C)
J.M.	M	66	Arteriosclerosis, myocardial fibrosis, sclerotic aorta. Myocardial infarction in 1940. Diabetes mellitus	11 (S) 18 (C)	11 (C) 16 (S)
S.K.	F	66	Arteriosclerosis, hypertension, enlarged heart, myocardial fibrosis. ECG: Bundle branch block	16 (S)	17 (C)
S.S.	M	65	Arteriosclerosis, enlarged heart, myocardial fibrosis, dilated aorta. ECG: Myocardial damage	29 (C) 48 (S)	42 (S) 49 (C)
M.W.	M	70	Arteriosclerosis, enlarged heart, dilated aorta, myocardial, fibrosis. ECG: Myocardial damage. Myocardial infarction, 1938	30 (S)	11 (C)
F.P.	F	58	Arteriosclerosis, hypertension, enlarged heart, myocardial fibrosis. ECG: Myocardial damage	22 (S) 38 (C)	22 (C) 36 (S)
M.F.	M	47	Arteriosclerosis, myocardial fibrosis, aortic sclerosis. ECG: Myocardial damage. Myocardial infarction, 1942	26 (S) 39 (C)	22 (C) 39 (S)

* (S) Physiological saline.
† (C) Cytochrome C.

up or utilized by the tissues within 5 minutes. However, we were unable to demonstrate any increase in the capacity for effort without pain in patients with angina of effort following intravenous injections of 50 mg of Cytochrome C as compared with placebo injections of physiological saline solution.

16365

The Delay in the Action of Digitalis Glycoside (Lanatoside C.)*

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Since the studies of Clark[1] were published, it has been believed[2] that after the administration of digitalis glycosides, a latent period occurs before a cardiac effect is produced.

In our previous studies however, concerning the effect of different concentrations of Lanatoside C upon the embryonic duck heart, we observed that although the above described latent period did exist, nevertheless, its duration appeared to vary inversely with the

* Aided by grants from the Life Insurance Medical Research Fund and Sandoz Chemical Works, Inc.

1 Clark, A. J., *Proc. Roy. Soc. Med.*, 1912, **5**, 181.

2 Gold, H., Cattell, M., Modell, W., Kurt, N. T., and Kramer, M. L., *Proc.*, 1943, **2**, 80.

TABLE I.
Relationship Between Concentration of Digitalis Glycoside and Time of Occurrence of "Digitalis Effect" in Embryonic Duck Hearts.

Digitalis glycoside (mg/cc)	Diameter embryonic sinus (Mm)	Hearts (No.)	Onset: "digitalis effect" (Min.)
.00005	30	15	39
.0001	28	19	22
.0005	32	18	15
.001	33	39	7
.002	30	4	4
.005	30	8	3
.01	30	7	1.75
.05	31	5	0.50
.10	30	10	0.25

concentration of glycoside in contact with the heart. In these same studies however some delay in cardiac response was observed even at the greatest concentration of digitalis glycoside employed (0.001 mg per cc). It seemed important then to discover the effect, at higher concentrations of glycoside, upon the described latent period. The embryonic duck heart lent itself admirably for the purpose because of its relative paucity of tissue which allowed a possible opportunity for changing rapidly, the milieu of the cardiac cells.

Methods. The duck hearts were obtained as previously described[3,4] from embryos having a vascular sinus of 25-35 mm in diameter. The temperature of the fluid bathing the embryonic heart, was maintained at 35° C which caused more rapid beating than observed in previous studies. Consequently the appearance of A-V block or missing beats also occurred earlier. Either of these two latter abnormalities in rhythm were considered indicators of a "digitalis effect." Lanatoside C, varying in concentration from 0.00005 to 0.10 mg per cc of Tyrode's solution was employed in all experiments.

Results. As Table I indicates, the delay in "digitalis effect" varied inversely with the concentration of digitalis glycoside in contact with the embryonic hearts. For example, 18 hearts exposed to a concentration of 0.00005 mg of Lanatoside C per cc, beat for an average period of 39 minutes before exhibiting A-V block. On the other hand, hearts exposed to concentrations of 0.002 mg per cc or higher, almost immediately exhibited the acceleration of beating previously noted[3] in hearts affected by glycoside and exhibited A-V block much sooner. As a matter of fact, the embryonic hearts, exposed to 0.10 mg of glycoside per cc exhibited A-V block in 15 seconds or less. It is possible that the so-called latent period observed in previous studies may be explained as delay attendant to the penetration of digitalis glycoside into the cells of the adult heart.

Conclusion. The evidence obtained from the actions of Lanatoside C upon the embryonic duck heart indicated that such a heart exhibits a "digitalis effect" with no fixed latent period as described in previous studies. It would appear that if a sufficient quantity of digitalis glycoside is present, a "digitalis effect" may be expected almost immediately.

[3] Friedman, M., and Bine, R., Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 162.

[4] Friedman, M., and Bine, R., Jr., *Am. J. Med. Sci.*, 1947, **214**, 633.

16366

Studies of Fluorocardiography in Normal Subjects.

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In previous notes,[1,2,3] the patterns of the tracings which can be obtained in normal subjects by means of fluorocardiography (electrokymography) have been outlined. The present note reports supplementary observations which deal with the following problems: the tracing of the ascending aorta, the tracing of the pulmonary veins, and the velocity of the pulse wave in the pulmonary circulation. Also the origin and shape of the pulsations of the lung parenchyma are discussed.

Technique. The observations were made by using a Sanborn apparatus for fluorocardiography (electrokymography), and a Sanborn Stethocardiette for phonoelectrocardiography. As previously reported, fluorocardiography records on a continuous film, moving at the speed of 75 mm per second, the pulsations of various areas of the cardiovascular silhouette or the opacity changes of a pulmonary field on x-ray. This is made possible by the use of the x-ray machine, a phototube, a diaphragm with a slit-like opening, a small fluorescent screen, and various stages of amplification. The tracing is recorded by the galvanometer of the Stethocardiette while a simultaneously recorded phonocardiogram permits exact timing of the fluoroscopic pulsations.

The observations were made on 15 normal subjects between the ages of 16 and 30. A total of 10 observations of the pulmonary veins, 10 observations of the ascending aorta, and 15 observations on velocity of the pulmonary arterial wave were performed.

The ascending aorta was studied in the 10 degree and 45 degree left oblique positions. The slit was placed first over the lowest point above the shadow of the right auricle showing an arterial pulsation, then it was moved step by step toward the arch.

The pulmonary veins were studied by applying the slit transversely over an area about 2 cm beyond the convexity of the right auricle in the postero-anterior position. The lung markings are essentially due to the shadows of the pulmonary vessels. The patterns of the arterial and venous ramifications are generally identical, except for the hilar regions; therefore, arterial and venous shadows cannot be identified as such on the routine roentgenograms. The stronger arterial pulsations overshadow the weaker venous pulsations; therefore the hilar pulsations, visible with the naked eye in certain instances (hilar dance), and the pulsations of the pulmonary parenchyma recorded by fluorocardiography, are mainly arterial. However, the arrangement of the larger vessels in the hilar regions is different for the arteries and veins. It has been known that veins coming from the middle and lower lobes on the right, in their almost horizontal course, cross the downward running arteries perpendicularly and stand out within the bright band of the lower trunk bronchus; they are visible sometimes on routine chest roentgenograms (Assman[4]) and more often on laminograms taken at the appropriate depth (Chatton and Maleki[5]).

Marchal[6] identified these as vascular shadows of pulmonary veins by injecting an opaque medium intravenously and observing a definite time difference of its arrival at the hilar arteries and at these vessels. These

1 Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 336.

2 Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 348.

3 Luisada, A. A., and Fleischner, F. G., Proc. Soc. Exp. Biol. and Med., 1948, **66**, 436.

4 Assman, H., *Die Klinische Röntgendiagnostik der Inneren Erkrankungen*, 5th Ed., Vogel, Berlin, 1934.

5 Chatton, P., and Maleki, A., *J. de Radiol. et d'Electr.*, 1947, **28**, 285.

6 Marchal, M., *Arch. Mal. Coeur*, 1946, **39**, 345.

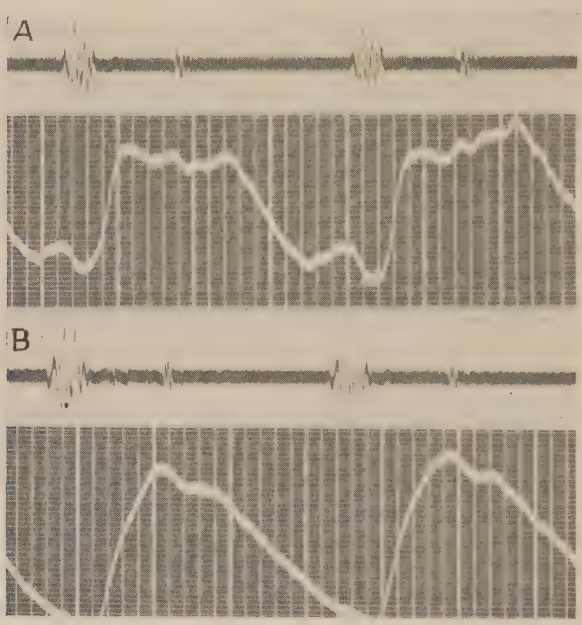

FIG. 1.
Aortic tracings in a normal adult in a 10° left anterior oblique position. (A) Ascending aorta. (B) Aortic arch.

faint vascular shadows can be identified only occasionally by fluoroscopy. However, information gained from Herrnheiser's detailed studies[7] on the hilar shadows simplified the procedure. In addition to these horizontally crossing veins (*Vena costalis* of the middle lobe and *Vena apicohorizontalis* of the lower lobe), the *Venae basalis posterior* and *axillo-basalis posterior* cross the bright cardio-hilar interspace obliquely, while only one artery of sizable caliber is found in this area. This being an area with predominantly venous vascular shadows, tracings were obtained by us with unexpected frequency, which presented a distinct venous characteristic, namely a presystolic positive wave.

To identify this venous pattern and to rule out incidental waves due to positional movements into and out of the field of observation of arteries or other structures, the following test was performed. The slit was placed vertically upon the border of the right auricle. In successive steps of a few millimeters each, the slit was moved laterally across the bright cardio-hilar interspace, the final tracing being taken with the slit right upon the arterial hilar trunk. In addition, routine tracings of the right auricle in the postero-anterior view and the left auricle in right oblique view were taken. Thus it became evident that the positive presystolic wave recorded in the "venous field" corresponded temporally to the presystolic left auricular contraction; moreover, there was no evidence of any other pulsatory or positional deflections which might have caused this wave.

The velocity of the pulse waves in the lesser circulation was studied in the following way. Comparison was made of the tracings obtained by placing the slit first over the pulmonary knob, then over the right hilar shadow, and later at the base of the right lung, or better at the lowest visible point above the right diaphragm, following the previously indicated technique.[1] Measurements made in 50 chest films of adults of various sizes and habitus gave the following average data: (a) Distance between pulmonary knob and right hilar shadow, 8 cm. (b) Distance between right hilar shadow and visible base of right lung, 11 cm.

[7] Herrnheiser, G., *Am. J. Roentg.*, 1942, **48**, 595.

Results and Comments. (1) *The tracing of the ascending aorta* presents a typical pattern which is different from that of tracings recorded over the aortic arch. Its features are as follows (Fig. 1): early systolic drop; rapid rise; early peak; only slight descent (or no descent) during the second half of systole: small incisura; high and occasionally prolonged wave after the incisura; this wave may be higher than the main wave in some cases. This typical tracing resembles certain tracings of pressure recorded by Tigerstedt[8] on the carotid artery of rabbits while it differs from those recorded by the same author and by Wiggers[9] in dogs and also from the reconstruction of aortic pressures by Hamilton and Dow.[10] Intra-aortic pressure, recorded by Marey[11] in the horse, again presents a more conventional profile. This indicates that the pattern recorded by us in normal human subjects is not merely caused by volume changes (parallel to pressure changes) but is also markedly affected by motions of the heart and vessels. The lowering of the aortic root by ventricular systole and the medial displacement of the ascending aorta by rotation of the heart in the same phase apparently reduce the height of the aortic wave. This is confirmed by the initial drop of the tracing. Opposite movements, taking place in diastole, add their effect to that of the dicrotic wave and create a high wave on the tracing. The proximity of the ascending aorta to the left ventricle may contribute to the fact that in some subjects the profile of the aortic pulse during systole greatly resembles a tracing of intraventricular pressure.

(2) *Tracing of the Pulmonary Veins.* Tracings recorded in the right intercardio-hilar space as suggested by Marchal[6] and discussed above show close relation to those of the auricles. They reveal a presystolic positive wave (increased volume) synchronous with the negative wave (contraction or decreased volume) of the left auricular tracing. Such unequivocal records were not obtained in every single case, apparently because of anatomical variability and resulting technical difficulty in placing the slit at the right spot.

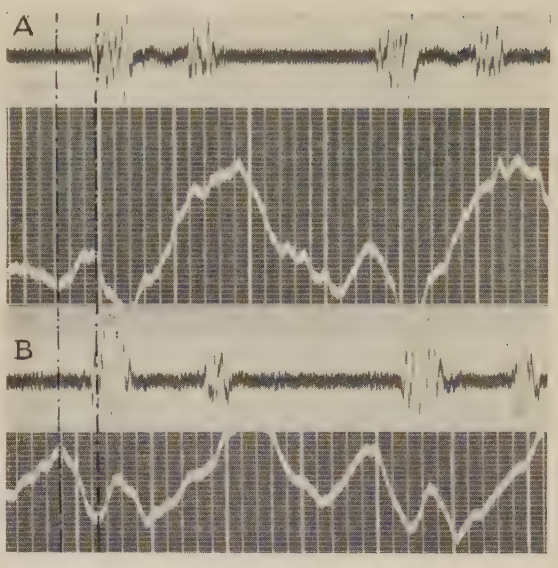

FIG. 2.
Tracing of the pulmonary veins in postero-anterior position (A) compared with that of the left auricle in left oblique (B). Normal subject. The presystolic drop of the auricular tracing is matched by a presystolic rise in the venous tracing.

(3) *Velocity of the pulse wave in the pulmonary circulation.* While examples of the tracings are given in Fig. 3, the average data obtained in 10 subjects are summarized in the following table. This also gives the maximum and minimum variations and the range of physiological variations on the basis of 8 cases out of 10, disregarding exceptional variations.

For greater accuracy, the distances between 1st sound and foot of the pulse waves were measured by using the beginning of the 1st sound vibrations but not that vibration which marks the opening of the semilunar valves.[12] This gives a prolongation of the absolute individual temporal relationship of about 0.05 seconds. However, it fails to change the

8 Tigerstedt, R., *Die Physiologie des Kreislaufes,* 2nd Ed., DeGruyter, Berlin, 1922, **3**, p. 218.

9 Wiggers, C. J., *Modern Aspects of the Circulation in Health and Disease,* Lea and Febiger, Phila., 1923.

10 Hamilton, W. F., and Dow, P., *Am. J. Physiol.,* 1939, **125**, 48.

11 Marey, E. J., *La Méthode Graphique dans les Sciences Expérimentales,* Masson, Paris, 1885.

12 Rappaport, M. B., and Sprague, H. B., *Am. Heart J.,* 1942, **23**, 591.

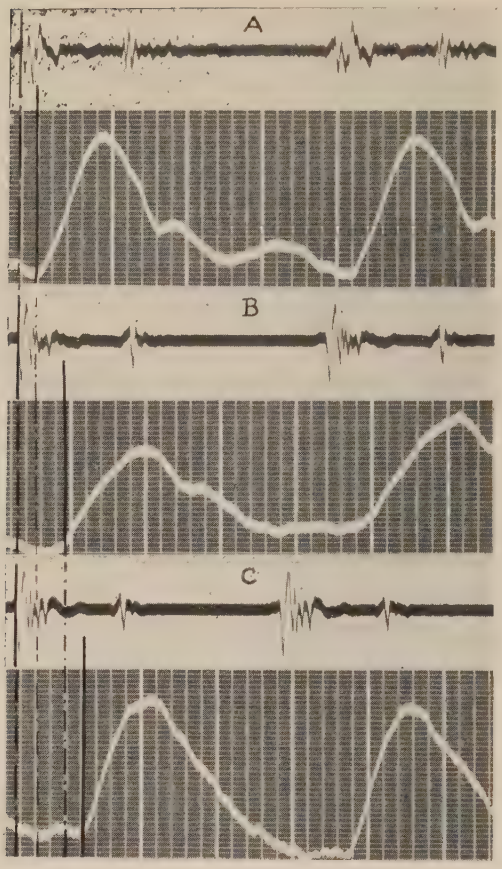

FIG. 3.

Tracing of the pulmonary knob (A), of the right hilar shadow (B), and of the visible base of the right lung (C) in a normal subject. Postero-anterior position.

relative intervals because all figures are obtained in the same way. As indicated in the table, the average times of arrival of the pulse waves are 0.08 sec. for the pulmonary knob, 0.12 sec. for the right hilus, and 0.16 sec. for the visible base of the right lung. Using the average figures of 8 and 11 cm indicated above, we obtain a velocity of the pulse waves which is 2 meters per second between pulmonary knob and right hilus and 2.75 meters per second between right hilus and visible base of the right lung. These speeds should be compared with the known speeds of the pulse waves in the greater circulation. These vary between 5 and 9 meters per second in normal subjects and are greater in the peripheral vessels (7.5) than in the aorta (4.5).[13,14]

Therefore, while the speed of the pulse in the lesser circulation is lower than in the greater, being roughly one third of the latter, the pulse increases its speed in the small, less extensible arterioles both in the lesser and in the greater circulation. The lower velocity of the pulse wave in the pulmonary circulation is caused by the lower pressure and by the greater extensibility of the vessels. This fact had been demonstrated in experimental animals and appears now confirmed for man.

(4) *Shape and cause of the pulmonary wave.* The peak and the shape of the pulsatory wave of the lung have been the object of special attention. The peak has a less fixed position in the tracing of the pulmonary parenchyma than in that of the hilus. In spite of that, it is easy to see that the peak in the pulmonary tracing is nearer to the foot than in the hilar tracing; it is followed by a more rapid descent; it often follows closely that small vibration of the 2nd sound which marks the opening of the mitral valve.[15] For these reasons, we came to the conclusion that the tracing of the pulmonary parenchyma is similar to a plethysmogram and is modified by both arterial and venous changes in the blood content of the lung. The rise and the ascending part of the wave are of arterial origin, as shown by the respective time relationships that they have with similar waves of the hilar tracing and of the tracing of the pulmonary knob. On the contrary, the descending branch of the wave reflects mainly changes in venous tracing. It is possible that occasionally some venous component is present in the hilar tracing too but this is less likely for anatomical reasons and because usually much less amplification is required for recording that tracing. Therefore, possible venous components are minimized by the technique used.

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[13] Bazett, H. C., and Dreyer, N. B., *Am. J. Physiol.*, 1922, **63**, 94.

[14] Wiggers, C. J., *Ann. Int. Med.*, 1932, **6**, 12; *Am. Heart J.*, 1938, **16**, 515.

[15] Hamilton, W. F., Circulation Through Special Regions, in *Howell's Textbook of Physiology* edited by J. F. Fulton, W. B. Saunders, Phila., 1946.

TABLE I.
Velocity and Duration of Pulmonic Arterial Waves.

	Avg distance from 1st sound to foot of pulse wave	Distance between foot and peak of the same wave	Transmission time	Speed of the wave
Pulmonary knob	0.08 sec. ± 25% Mx = 0.10 Mn = 0.05	0.14 sec.		
Right hilar shadow	0.12 sec. ± 25% Mx = 0.15 Mn = 0.09	0.24 "	Pulm. knob to rt. hilus 0.04 sec.	2 M/sec.
Right lung (visible base)	0.16 sec. ± 25% Mx = 0.22 Mn = 0.10	0.23 "	Rt. hilus to visible base of rt. lung 0.04 sec.	2.75 M/sec.

Summary. A study of 15 normal subjects has been performed by using fluorocardiography, a method which permits recording on a continuous film of the pulsations of various cardiovascular structures revealed by the x-ray. (a) The tracing of the ascending aorta presents a typical pattern which partly is due to transmission of intraventricular pressure and partly to motions of the aortic root connected with ventricular systole and diastole. (b) It is possible to record in certain cases a tracing of the pulmonary veins. Its most typical feature is a positive wave during presystole. (c) The velocity of the pulse wave in the lesser circulation is much lower than in the greater circulation. It is more rapid in the smaller branches than in the stems of the pulmonary artery. Average figures are given. (d) The tracing of the pulmonary parenchyma is the equivalent of a plethysmogram. While the rise and the ascending branch of the wave are of arterial pulmonary origin, the peak and the descending branch of the wave are probably of venous pulmonary origin.

16367

Bacillomycin: An Antibiotic from *Bacillus subtilis* Active against Pathogenic Fungi.*

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Since the discovery of *subtilin* by Jansen and Hirschmann,[1] 3 additional antibiotics i.e., *bacitracin, bacillin,* and *eumycin* have been described as products of strains of *Bacillus subtilis* (for an excellent, comprehensive review see Benedict and Langlykke[2]). With the exception of *eumycin,* the antibiotics possess incidental antifungal properties in association with a more specific antibacterial action.

* The authors wish to express their indebtedness to Dr. E. G. Snyder and R. W. Whitley for purification and chemical studies; and to Miss Charlotte Campbell, Army Medical Department Research and Graduate School, for the systemic fungi spectrum.

† Present address: Army Medical Department Research and Graduate School, Washington, D.C.

1 Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.,* 1944, **4**, 297.

2 Benedict, R. G., and Langlykke, A. F., *Ann. Rev. Microbiol.,* 1947, **1**, 193.

TABLE I.
Antifungal Spectrum of Bacillomycin.

Organism	Source of strain	mg of Bacillomycin per ml of medium									
		0.5	0.25	0.10	0.05	0.025	0.010	0.005	0.0025	0.001	Control
Dermatophytes:											
Epidermophyton floccosum	Univ. of Penn.	—	—	—	—	—	+	+	+	+	+
Microsporum audouini	,, ,, ,,	—	—	—	—	—	+	+	+	+	+
Microsporum gypseum	,, ,, ,,	—	—	—	—	—	+	+	+	+	+
Trichophyton mentagrophytes	A.T.C.C.	—	—	—	—	—	+	+	+	+	+
Trichophyton rubrum	,,	—	—	—	—	—	+	+	+	+	+
Trichophyton schoenleini	Univ. of Penn.	—	—	—	—	—	+	+	+	+	+
Systemic Fungi:											
Blastomyces dermatitidis (Mycelial)	Duke Univ.	—	—	—	—	—	—	R	+	+	+
*Blastomyces dermatitidis** (Yeast form)	,, ,,	—	—	—	—	—	—	—	—	+	+
Candida albicans	Army Med. Sch.	—	—	—	—	R	+	+	+	+	+
Coccidiodes immitis	,, ,, ,,	—	—	—	—	—	R	+	+	+	+
Torula histolytica	Naval Med. Center	—	—	—	—	R	+	+	+	+	+
Histoplasma capsulatum	Duke Univ.	—	—	—	—	—	R	+	+	+	+
Hormodendrum pedrosoi	Univ. Brazil	R	R	+	+	+	+	+	+	+	+
Monosporium apiospermum	Duke Univ.	—	—	—	—	—	+	+	+	+	+
Nocardia asteroides	Army Med. Sch.	+	+	+	+	+	+	+	+	+	+
Phialophora verrucosa	Nat. Inst. Health	R	+	+	+	+	+	+	+	+	+
Sporotrichum schenckii	Duke Univ.	—	—	—	—	R	+	+	+	+	+
Blastomyces braziliensis	,, ,,	—	—	—	—	—	+	+	+	+	+

Cultures were incubated at 30°C for 15-17 days. Medium was Sabouraud maltose agar.
* Inoculated from a culture in "yeast" phase and incubated at 37°C for 20 days.
Key: + = growth, R = growth restricted; — = no growth.

In the course of a program designed to find antibiotics active against *Torula histolytica* infections, there was isolated from a contaminated *Actinomyces griseus* culture a strain of *Bacilius subtilis* which possessed negligible antibacterial activity and striking fungistatic properties. Although concentrates of this antibiotic and *eumycin*[3,4] showed a spectrum similar in many respects, the limited available data on the chemistry of *eumycin* as well as its specific action on *Corynebacterium diphtheriae* and acidfast bacilli appeared to differentiate it from our antibiotic and warranted further study. The antibiotic substance has been designated *bacillomycin.*

Bacillomycin occurs in the cell-free fermentation liquor after cultivation of the organism for 2 to 3 days in shake culture or 5 to 6 days in surface culture at 24-28°C in a synthetic medium composed of 20 g glucose, 5 g l-glutamic acid, 0.5 g $MgSO_4$, 0.5 g KCl, 1 g KH_2PO_4, 0.15 mg $FE_2(SO_4)_3 \cdot 6H_2O$, 5.0 mg $MnSO_4 \cdot H_2O$, 0.16 mg $CuSO_4 \cdot 5H_2O$ and 1000 ml distilled water; final pH is 6.0. Maximal yields of the product are obtained with a rise in pH of the medium to 7.0-7.6.

Crude filtrates are assayed against a standardized spore suspension of *Trichophyton mentagrophytes* by the agar cup method on Sabouraud's maltose agar. Standard solutions and experimental samples are tested in duplicate on each plate and the latter incubated at 30°C for 72 hours. The unit of activity is equivalent to the zone produced by 0.1 mg of a standard preparation of *bacillomycin.* This represents a zone of 20 to 25 mm.

The antifungal activity of partially purified *bacillomycin* was measured by the agar plate-dilution method. The results are reported in terms of the smallest amount of the antibiotic in a constant volume of test medium (10 ml) which will give complete inhibition of the test organism. Incubation of the tests (with one exception) was at 30°C for 15-17 days. A summary of the inhibition experiments on a spectrum of pathogenic fungi is compiled in Table I.

Bacillomycin is precipitated from the broth by acidifying to pH 2.5 with hydrochloric acid solution. The precipitate is extracted with ethanol, washed with ether and dried *in vacuo* over phosphorus pentoxide. (The ether step assumes importance in the extraction since an additional antibiotic elaborated by the organism and active against gram positive bacteria is also precipitated at pH 2.5. However, whereas the antifungal product is largely ether insoluble, the antibacterial activity is soluble.) The activity averages 5 units/mg and the yield is approximately 0.3 g/liter broth with a 35 to 50 % recovery.

Bacillomycin is soluble in methanol, ethanol, n-butanol and acetone. It is precipitated by concentrated neutral ammonium sulfate solution. The active substance is readily adsorbed on activated charcoal but is difficult to elute. The antibiotic will not dialyze through cellophane membranes.

Bacillomycin is not destroyed by trypsin or pepsin. The product is stable in dried form. Samples stored at room temperature have retained their activity for months. Solutions adjusted in pH to include acid (3.0) neutral and alkaline (9.0), may be autoclaved (120°C-20 minutes) with no appreciable loss of activity.

Summary. A strain of *Bacillus subtilis* has been isolated which elaborates a previously undescribed antibiotic "Bacillomycin." This antibiotic possesses striking antifungal activity and almost complete lack of antibacterial action. The fungus spectrum of "Bacillomycin" includes practically all of the important dermatophytes and systemic fungi. A satisfactory bioassay employing the agar cup plate technic and a spore suspension of *Trichophyton mentagrophytes* is described. Some physical and chemical properties of the antibiotic are given together with a simple procedure for its concentration from culture broths.

[3] Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

[4] Burdon, K. L., and Johnson, E. A., Conference on Antibiotic Research, Antibiotics Study Sect., Nat. Inst. Health, 1947.

16368

Renal Tubular Secretion of Potassium in the Normal Dog.*

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During the administration of salyrgan† (sodium salt of mercury salicyl-allylamide-ortho-acetate) to dogs, it was observed that the rate of potassium excretion frequently became constant and remained at a fixed level despite marked changes in the calculated rate of potassium filtration at the glomerulus. A constant excretory rate dissociated from filtered load is strongly suggestive of a tubular secretory mechanism. Such a mechanism for the addition of potassium to the tubular urine has, in fact, been demonstrated in the dog by experiments to be described.

Material and Methods. Experiments were performed on 4 trained, unanesthetized female dogs. To obtain stable plasma creatinine and inulin concentrations and to assure constant rates of potassium intake, solutions were administered by continuous infusion. Urine and heparinized venous blood samples‡ were collected by the usual techniques for the determination of clearances.

The plasma creatinine clearance was used as a measure of glomerular filtration rate. The equivalence of the creatinine clearance and filtration rate in the dog is generally accepted. In 2 experiments inulin clearances were simultaneously determined to check on the validity of the creatinine clearance as a measure of filtration rate under the circumstances of these experiments.

Creatinine was determined in tungstic acid filtrates of plasma and in diluted urine by a modification of the Folin method.[1] Inulin was determined by a modification of Harrison's method.[2] Both plasma and urine were treated with yeast before precipitation with zinc.

Potassium and sodium in plasma and urine were determined with an internal standard flame photometer,[3] after addition of a standard amount of lithium to each specimen and dilution. The error of the method in our hands does not exceed 1% in the recovery of added amounts. The presence of protein in diluted plasma samples did not interfere with the determination since ashed specimens gave results identical with those obtained by simple dilution. The addition of amounts of sodium greater than those present in plasma samples was found not to affect potassium determinations.

Results. An experiment showing the effect of salyrgan on potassium excretion is summarized in Table I. Soon after the administration of salyrgan the rate of excretion of potassium reached a value of about 45 μeq/min and remained at this level for a period of more than 2 hours despite a fall of 35% in the filtered potassium and during marked changes in urine flow and sodium excretion. Similar results were obtained in each of 3 other dogs. A slight rise in potassium excretion with time was sometimes observed in

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† The salyrgan used in this study was supplied by the medical research department of Winthrop-Stearns, Inc.

‡ Arterial blood samples were simultaneously obtained in several instances. The concentration of potassium in arterial plasma did not differ measurably from that in venous plasma during the infusion of KCl.

1 Shannon, J. A., and Fisher, S., *Am. J. Physiol.* 1938, 122, 765.

2 Harrison, H. A., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 111.

3 Berry, J. W. Chappell, D. G., and Barnes R. B., *Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 605

TABLE I.
Effect of Salyrgan on Potassium Excretion. Dog F. 13.9 Kilos.

Clearance period	Time, min.	Plasma concentration*			Urine flow, ml/min	Creatinine clearance, ml/min	"Filtered"†		Excreted	
		Creatinine, mg %	Sodium, meq/L	Potassium, meq/L			Sodium, μeq/min	Potassium, μeq/min	Sodium, μeq/min	Potassium, μeq/min
	0		148	3.7						
	Priming creatinine 0.9 g in 200 ml isotonic NaCl.									
	Start infusion 0.45% creatinine in isotonic NaCl at 2 ml/min.									
1	18-38	10.8	149	3.6	0.62	59	8800	212	105	20
2	38-59	11.6	149	3.5	0.72	58	8600	203	93	19
	62		Salyrgan 1 ml I.V. 3.2 ml Salyrgan added to 400 ml of infusion.							
3	81-104	12.6			8.89	67	10000	228	1410	31
4	104-115	12.5	149	3.4	10.03	81	12100	275	1860	44
5	115-131	12.3			6.60	69	10300	234	1270	48
6	180-196	13.8			2.12	53	7800	175	350	45
7	196-211	14.5	148	3.3	2.23	52	7700	172	353	45
8	211-232	—			2.90	—	—	—	440	46

* Plasma samples obtained at midpoint of corresponding clearance periods.
† Plasma concentration times creatinine clearance; uncorrected for Donnan equilibrium.

these experiments. The rate of potassium excretion after salyrgan was nearly constant in each experiment but varied from dog to dog, 45 μeq/min being the lowest rate observed while 150 μeq/min was the highest. Salyrgan did not always effect an increase in potassium excretion. When potassium excretion was initially increased by administration of KCl, salyrgan produced a decrease in the excretion rate.

The most direct evidence for a secretory mechanism for potassium would be the demonstration of more potassium in the urine than could be accounted for by glomerular filtration. Since the amount secreted would probably be small in relation to the amount which might be filtered, it was to be expected that some difficulty would be encountered in demonstrating a secretory mechanism. However, in each of the 4 dogs it has been possible to obtain at least one experiment in which the excreted potassium exceeded that filtered. Only in the dog with the largest potassium excretion after salyrgan was this achieved in the first attempt. In the other dogs several preliminary experiments were sometimes necessary to determine the optimum infusion rate for demonstration of this phenomenon. Achievement of the necessary conditions seemed to be facilitated by the preliminary oral administration of KCl[4] for a week before the experiment and by the administration of the KCl during experiments in hypertonic solution and at a moderate rate. One such experiment is shown in Table II. In this experiment, within 40 minutes of the time that the infusion rate was increased to 0.67 meq/min, the excreted potassium exceeded that "filtered" by 25% and ratios of excreted to "filtered" varied between 1.15 and 1.33 in 9 successive clearance periods. Inulin clearances were determined in 2 similar experiments, one of which is shown in Table III. In both experiments the inulin clearances corresponded closely with simultaneously determined creatinine clearances. In the 2 other dogs, ratios of excreted to "filtered" potassium greater than one were obtained

4 Thatcher, J. S., and Radike, A. W., *Am. J. Physiol.*, 1947, **151**, 138.

TABLE II. Effect of Infusion of Hypertonic KCl. Dog D.† 18.6 Kilos.

Clearance period	Time, min.	Plasma* conc. Creatinine, mg %	Plasma* conc. Potassium, meq/L	Urine flow, ml/min	Creatinine clearance, ml/min	"Filtered" K,‡ μeq/min	Excreted K, μeq/min	Ratio: Excreted / "Filtered" K
	0	Priming creatinine 1.85 g in 20 ml water.						
	2	Start infusion 1.7% creatinine in 0.33 N KCl at 1 ml/min.						
	5		5.0					
1	59-78	24.8	5.5	1.05	63	346	194	0.56
2	78-104	24.9	5.5	0.84	64	352	173	0.49
3	104-124	25.7	5.4	1.17	63	340	241	0.71
	125	Increase potassium concentration of infusion to 0.67 N.						
4	164-184	25.1	6.0	2.76	75	450	556	1.24
5	184-205	25.1	6.5	2.91	76	494	615	1.25
6	205-226	25.7	7.1	2.85	74	525	625	1.19
7	226-245	25.7	7.5	3.09	76	570	670	1.18
8	245-266	25.1	6.8	3.26	78	530	702	1.33
9	266-291	24.8	6.7	2.90	78	522	678	1.30
10	291-311	25.1	7.4	2.95	77	570	665	1.17
11	311-336	25.1	7.6	3.09	79	600	715	1.19
12	336-355	24.8	7.8	2.89	80	624	720	1.15

* Plasma samples obtained at midpoint of corresponding clearance periods.
† Dog received 5 g KCl twice daily by mouth for one week before this experiment.
‡ Plasma concentration times creatinine clearance; uncorrected for Donnan equilibrium.

TABLE III. Dog M*. 11.8 Kilos.

Clearance period	Time, min.	Plasma† conc. Creatinine, mg %	Plasma† conc. Inulin, mg %	Plasma† conc. K, meq/L	Urine flow, ml/min.	Clearance Creat., ml/min.	Clearance Inulin, ml/min.	Clearance ratio: Inulin / Creat.	"Filtered"‡ K, μeq/min.	Excreted K, μeq/min.	Ratio: Excreted / "Filtered" K
	−1			4.2							
	0	Priming creatinine 1.1 g; inulin 0.6 g.									
	2	Start infusion 0.9% creatinine, 0.8% inulin in 0.35 N KCl at 1.2 ml/min.									
1	60-83	21.2	20.8	5.0	1.62	60	58	0.97	300	298	0.99
2	83-112	20.6	19.4	4.9	1.48	58	59	1.02	284	294	1.04
3	112-132	20.4	19.2	4.7	1.49	61	59	0.97	286	318	1.11
4	132-152	20.1	18.4	5.1	1.66	60	61	1.02	306	352	1.15
5	152-172	19.5	18.0	5.2	1.89	62	60	0.97	322	385	1.20
6	172-194	19.1	17.4	5.3	1.93	62	63	1.02	328	398	1.21
7	194-216	18.9	17.2	5.5	1.94	64	64	1.00	352	420	1.19

* Dog received 5 g KCl twice daily by mouth for 12 days before this experiment.
† Plasma samples obtained at midpoint of corresponding clearance periods.
‡ Plasma concentration times creatinine clearance; uncorrected for Donnan equilibrium.

in 22 clearance periods in 3 experiments; the ratios reached at least 1.15 in each dog. It is worthy of note that after preparation with oral KCl high rates of potassium excretion were attained with only minimal elevation of the plasma potassium concentration (Table III).

Discussion. The excess of excreted potassium over filtered load was well beyond the limits of experimental error. There is no reason to believe that the filtration rate actually exceeded the creatinine clearance, especially since the inulin and creatinine clearances were the same.

It should be noted that the amount of potassium filtered has been calculated simply as the product of creatinine clearance and plasma potassium. Correction of the filtered potassium for the Donnan equilibrium would lower the filtered load by about 5% and increase the excess of excreted potassium observed in these experiments.

The data obtained constitute evidence for the existence of a mechanism for the addition of potassium to the tubular urine.§ The coexistence of tubular mechanisms for both reabsorption and secretion of a single substance has not previously been demonstrated. Tubular secretion of a number of substances generally considered to undergo only filtration and reabsorption, including potassium, has been suggested by Barclay, Cooke and Kenney,[6] but the evidence on which this conclusion is based is not presented in the published abstract.

Summary and Conclusions. A constant rate of potassium excretion, dissociated from filtered load, occurring after salyrgan administration suggested a tubular secretory mechanism located, presumably, in the distal tubule. The presence of such a mechanism has been demonstrated by the intravenous administration of hypertonic KCl solutions which yielded rates of potassium excretion considerably above the rates of filtration of potassium at the glomerulus.

§ Doctors Mudge, Foulks, and Gilman[5] inform us that they have similarly concluded that potassium is secreted by the renal tubules on the basis of observations during forced osmotic diuresis.

[5] Mudge, G. H., Foulks, J. G., and Gilman, A., personal communication.

[6] Barclay, J. A., Cooke, W. T., and Kenney, R. A., XVII Internat. Physiol. Congress, Abstracts of Communications, 1947, p. 58.

16369 P

The Renal Excretion of Potassium.*

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The potassium (K) excreted in the urine under normal circumstances can be accounted for by the tubular rejection of approximately 10% of the calculated filtered K. This has been accepted as evidence that K is excreted by the process of filtration and incomplete reabsorption. The possibility of tubular secretion has been suggested by isolated observations. McCance and Widdowson[1] reported a case of alkalosis and dehydration with a low filtration rate and a K clearance greater than the inulin clearance. Keith, King and Osterberg[2] noted similar K : Inulin clearance

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† National Research Council Fellow.

[1] McCance, R. A., and Widdowson, E. M., *Lancet*, 1937, **2**, 247.

[2] Keith, N. M., King, H. E., and Osterberg, A. E., *Arch. Int. Med.*, 1943, **71**, 675.

ratios in normal subjects receiving KCl, but gave no experimental data and did not mention possible K secretion in subsequent reports.[3] Barclay, Cooke and Kenney[4] stated that K, urea and phosphorus were excreted by the triple process of filtration, reabsorption and secretion, but gave no experimental results. Winkler and Smith[5] observed a maximum excretion of 35% of filtered K in dogs, but noted no evidence of tubular secretion. This preliminary report is submitted because in experiments on dogs we have repeatedly observed K clearances greater than the simultaneously determined filtration rates. In studies on trained dogs infused with KCl, Berliner and Kennedy[6] have also independently observed K excretion in excess of the calculated amount filtered.

Methods. Mongrel dogs were studied under light pentobarbital anesthesia. Conventional clearance techniques and analytical procedures were employed. Creatinine or thiosulfate clearances were used as a measure of the glomerular filtration rate. K was determined by an improved type internal standard flame photometer with an accuracy of ± 1%. K clearances were calculated without correction for the Donnan equilibrium or protein binding.

Results. In studies on the electrolyte excretion associated with urea diuresis, K clearances increased from 5-10% of the creatinine clearance at normal rates of urine flow to 80-90% of the creatinine clearance during marked diuresis with minute urine volumes of 18 to 35 cc and at creatinine U/P ratios of approximately 2. At lower creatinine U/P ratios the C_K/C_{cr} ratio exceeded 1 by as much as 0.36 without the administration of exogenous K. Under these circumstances the urea clearance/creatinine

TABLE I.
Effect of Urea Diuresis on the U/P Ratios of Creatinine and Potassium.

Elapsed time, min.	KCl infusion rate, mEq/min.	Urine vol., cc/min.	Serum K, mEq/L	Serum urea, mOsM/L	C urea / C_{Cr}	U/P Cr	U/P K	U/P K / U/P Cr
0		1.7	4.4	2.9	.58	27.9	2.4	.09
10	KCl infusion started.							
20		8.2	10.3	2.3	.72	7.6	4.6	.60
160	.450							
161	50% urea infusion started at 12.5 mOsM/min.							
180	.450	9.2	10.4	15.8	.66	5.02	3.7	.74
235	.350	—	10.6	—	—	1.41	1.53	1.09
255	.163	22.5	7.3	276	.88	1.82	2.37	1.30
275	.300	12.2	6.9	247	.81	2.15	2.99	1.39
295	.300	14.4	8.9	326	.85	1.33	1.60	1.20
310	.500	—	10.6	—	—	1.34	1.50	1.12

[3] Keith, N. M., and Osterberg, A. E., *J. Clin. Invest.*, 1947, **26**, 773.

[4] Barclay, J. A., Cooke, W. T., and Kenney, R. A., *XVII Internat. Physiol. Congress, 1947, Abstracts*, page 58.

[5] Winkler, A. W., and Smith, P. K., *Am. J. Physiol.*, 1942, **138**, 94.

[6] Berliner, R. W., and Kennedy, J. P., personal communication.

TABLE II
Effect of $NaHCO_3$ on the Renal Clearance of Potassium and Thiosulfate.

Elapsed time, min.	Urine vol., cc/min.	Serum K, mEq/L	Urine pH	Clearance S_2O_3 cc/min.	Clearance K cc/min.	$\frac{C_K}{C_{S_2O_3}}$
—143	Start of constant infusion .375 mEq KCl/min., maintained during entire experiment.					
0-20	3.8	7.3	5.6	56.8	67.5	1.19
40-60	1.8	6.9	5.6	55.7	53.4	.96
80-100	2.0	5.8	5.4	52.7	49.4	.94
120-140	2.6	6.3	5.6	51.2	46.8	.92
150-170	3.1	6.7	5.6	53.1	47.7	.90
	I.v. infusion of 200 mEq $NaHCO_3$ in 1 L. in 35 min.					
210-230	15.7	5.0	7.8	54.0	65.8	1.22
235-255	8.5	4.7	7.8	52.6	67.3	1.28
260-280	6.6	4.7	8.1	55.5	60.3	1.09

clearance ratios averaged .83 and never exceeded 1.0. In the sample protocol (Table I) both K and urea were infused and due to ECG changes the K infusion rate was not constant and therefore "spot" U/P ratios were taken.

Because of the elevation of urine pH occurring during marked urea diuresis and because of the observation of Darrow[7] that alkalosis is associated with K depletion when renal function is normal, the effect of alkalosis was studied in dogs receiving KCl by intravenous infusion. Under these conditions K clearances greater than filtration rates were also demonstrated (Table II).

C_K/C_{cr} ratios above 1 were observed in 7 different dogs with filtration rates varying from 23 to 75 cc per minute. K excretion in excess of filtration was not observed in every experiment. Factors which favored C_K/C_{cr} ratios greater than 1 were: (1) a moderate, rather than marked elevation of serum K when KCl was infused, (2) a decline in filtration rate which usually accompanied extreme urea diuresis, and (3) the duration of the KCl infusion. The first two factors would help reveal a secretory mechanism in the face of partial reabsorption of K. The third may represent a physiological adjustment.

Discussion and Conclusions. K clearances in excess of creatinine or thiosulfate clearances, under the conditions of these experiments, are interpreted as evidence that all of the K excreted in the urine cannot be accounted for by the filtration-reabsorption theory alone, but that tubular secretion of K also occurs. In the experiments on urea diuresis, urea clearances never exceeded creatinine clearances, and the ratios agreed with the observations of Shannon.[8] It is a reasonable hypothesis that during urea diuresis the secretion of K becomes apparent because its normal tubular reabsorption is hindered. The present data are insufficient to define the mechanisms involved in the secretory process or to demonstrate the relative magnitude of the processes of filtration, reabsorption and secretion in the renal excretion of K. Further work is in progress.

7 Darrow, D. C., *J. Clin. Invest.*, 1946, **25**, 324.

8 Shannon, J. A., *Am. J. Physiol.*, 1938, **122**, 782.

16370

Differences in Sera of Human Subjects with Respect to Heteroagglutinins for Mouse Erythrocytes.*

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In attempting to demonstrate and induce agglutinins in the blood of 9 types of hybrid mice and in mice of 5 strains inbred for many generations, suspensions of mouse erythrocytes in human sera were routinely used as control material.[1] It was thus noticed that mouse erythrocytes were agglutinated by the sera obtained from human subjects of all 4 blood groups. There seemed to be no special affinity of the erythrocytes of mice for sera of any one type as such. It was apparent, however, that not all the samples of sera from human subjects possessed the property of agglutinating mouse erythrocytes. The negative reactions were heterospecific with respect to the blood groups in human subjects. The most obvious conclusion, therefore, was that there existed an antigen in mouse erythrocytes, the serological analog of which was present in most, but not all, human sera. Subsequent experiments showed that this serological component was specifically adsorbed by homologous antigen. For the sake of convenience, "Mo agglutinin" will henceforth be used to designate this component of human serum.

Gorer[2–5] demonstrated that isoagglutinins were present in the sera of mice in which tumors had recently regressed. This work was independently duplicated by Lumsden[6] who induced isoagglutinins in rats by means of normal or malignant nucleated cells. Both Gorer[3] and Lumsden[6] concluded that the natural resistance of a rat or a mouse to a tumor transplantation is directly proportional to the capacity of the animal to produce agglutinins.

In this paper we have been concerned with an agglutinin for mouse erythrocytes which occurs in most human sera and which is similar to or identical with that developing in mouse sera when transplanted tumors regress. While this is probably not related to the factors that govern susceptibility to cancer in the human subject, it is of interest that it was not present in approximately 14% of human subjects. It is the purpose of the present paper to present the work on the attempts to determine the approximate incidence and data on the adsorption and specificity of the Mo agglutinin in human sera.

Method and Materials. In these experiments, mice† of the following inbred strains were employed: C_3H, C_{57} black, I, A, and JK. The following types of hybrid mice of F_1 and F_2 generations were also used: $C_{57}xC_3H$, C_3HxC_{57}, C_3HxA, $C_{57}xCBAN$, $C_{57}CBANxA$, $C_{57}xA$, $AxCBAN$, $AxC_{57}CBAN$. In general, the technique used was similar to those described by Gorer.[2] The samples of human sera were obtained commercially, or from ourselves, our colleagues, and the Baltimore Rh Typing Laboratory. All sera were inactivated by heating in a water bath at 56°C for 30 minutes. 1:5000 merthiolate solution was used as a preservative.

The agglutination tests were carried out as follows: One drop of blood was taken from the mouse tail and suspended in 2 cc saline-citrate solution. The sera were diluted 1:2. Two drops of diluted serum and 4 drops of cell suspension were placed in a small test tube and mixed. The tubes were then centrifuged slowly for 30 seconds and then

* This work was aided by grants from the Anna Fuller Fund and the Donner Foundation.

1 Figge, F. H. J., Cohen, L., and Winokur, G., submitted for publication.

2 Gorer, P. A., *J. Gen.*, 1936, **32**, 17.

3 Gorer, P. A., *Brit. J. Exp. Path.*, 1936, **17**, 42.

4 Gorer, P. A., *Brit. J. Exp. Path.*, 1937, **18**, 31.

5 Gorer, P. A., *J. Path. and Bact.*, 1937, **44**, 691.

6 Lumsden, T., *Am. J. Cancer*, 1938, **32**, 395.

† All the mice used in these experiments were the progeny of mice obtained from Dr. L. C. Strong in 1941.

TABLE I.
Agglutination of Mouse AxCBAN (7) Erythrocytes with Various Human Sera.
(Numbers in parentheses are for identification of sera.)

A (1)	3+	A (57)	3+	B (21)	2+	AB (20)	3+	O (65)	2+
A (46)	2-3+	A (58)	2+	B (22)	—	AB (277)	3+		
A (50)	2+	A (59)	2+	B (23)	3+	AB (141)	3+		
A (51)	2-3+	A (60)	1+	B (24)	3+	AB (142)	—		
A (52)	3+	A (61)	2+	B (25)	2+	O (60)	2+		
A (53)	2+	A (62)	2+	B (26)	3+	O (61)	3+		
A (54)	—	A (63)	3+	B (27)	3+	O (62)	3+		
A (55)	2+	A (64)	3+	AB (1)	3+	O (63)	—		
A (56)	3+	B (1)	2+	AB (10)	3+	O (64)	—		

allowed to stand for 30 minutes after which they were read with the naked eye and checked microscopically. The readings were recorded as –, 1+, 2+, and 3+, depending on the degree of reaction.

The following adsorption technique was employed: 5 cc of oxalated blood of the desired type was centrifuged and the cells were washed 3 times with physiological saline solution. After the final washing, the supernatant saline was drawn off with a pipette and 10-12 drops of the corresponding serum to be used was added to the cells. The cells and serum were then mixed, centrifuged slowly for 30 seconds, and then allowed to stand 3 hours. The tube was then centrifuged at high speed for 5 minutes, after which the serum was removed and tested against a suspension of the blood cells used in the adsorption. If there was no agglutination of these cells, it was assumed that all the agglutinins had been removed from the serum.

Results. Human type A, B, AB, and O sera were found to agglutinate mouse erythrocytes. The agglutinogen was invariably present in the erythrocytes of mice of all of the 5 strains inbred and the 8 types of hybrid mice mentioned previously.

The data in Table I show that the Mo agglutinin was not present in 5 of 37 samples (approximately 14%) of human sera. It may also be observed that the negative reactions occurred in each of the four major blood groups so that the absence as well as the presence of the Mo agglutinin is not specifically associated with any of the standard human blood groups.

In the adsorption studies (Table II), the Mo agglutinin was shown to be a specific agglutinin which could be adsorbed from human serum by mouse erythrocytes, but not by human erythrocytes of any blood group (Table II). It was found to be entirely independent of α and β agglutinins as indicated by the fact that it was present in 4, but absent in 2, sera of the O group (Table I). Moreover, the α and β agglutinins could be adsorbed on the appropriate human erythrocytes without removing the Mo agglutinin. Conversely, the adsorption of the Mo agglutinin on mouse erythrocytes did not remove the α or β agglutinins or a heterospecific agglutinin for rabbit erythrocytes.[7,8] (Table II.)

The Mo agglutinin was also found to be present in both Rh+ and Rh– blood, and group A Rh+ cells failed to adsorb the Mo agglutinin. This data indicated that there was no apparent relationship between Mo agglutinin and Rh agglutinin. It is, perhaps, needless to point out that Rh sensitization is an induced phenomenon in Rh-persons who have had previous transfusions with Rh+ blood; or in some Rh– mothers who have been delivered of Rh+ children, the total incidence being comparatively low, at least in the latter group; whereas the Mo agglutinin is concerned with a hitherto unnamed naturally occurring agglutinin present in most, but not all, human sera examined thus far.

Discussion. It is necessary to clarify the contribution made by this work in relation to the advances made by others. Sievers[7] has presented a case of heteroagglutination with human serum and has shown that the agglutinins vary in different species of animals.

[7] Sievers, O., *Acta Path. et Microbiol. Scand.*, 1937, **14**, 553.

[8] Wiener, A. S., *Blood Groups and Transfusion*, 3rd ed., C. C. Thomas, Springfield, 1943.

TABLE II.
Cross Adsorption Experiments.
Specific Adsorption of Mo Agglutinin and Failure of Mouse Erythrocytes to Adsorb Rabbit Erythrocyte Agglutinins from Human Sera.

Human serum type	Erythrocytes					
	Human				Mouse	
	A-Rh+	A-Rh—	B	Rabbit	AxCBAN (7)	A (813)
A (50)	—	—	3+	2+	2+	
A (46) before adsorption	—	—	3+	3+	2-3+	
A (46) after adsorption with B cells	—	—	—	3+	2+	
B (22) before adsorption	3+		—	3+	—	
B (23) before adsorption	3+	3+		3+	3+	3+
B (23) after adsorption with A-Rh+ cells	—			3+	3+	3+
B (23) after adsorption with A-Rh— cells		—		3+	3+	3+
A (46) adsorbed with mouse (AxCBAN 7) cells	—	—	3+	3+	—	
B (23) adsorbed with mouse (C_3H 1534) vells	3+	3+	—	3+	—	

The original discovery of the Rh factor[8] was made by testing human beings with antiserum from rabbits which had been injected with Rhesus blood; in this fashion 85% of the individuals tested were positive. Gorer's[2] differentiation of the blood types in mice by reactions with human A serum was based on the quantitative or qualitative differences in the agglutinogens present in mouse erythrocytes. Since Gorer used himself as a source of type A serum, it is evident from his results that his blood contained Mo agglutinin. He apparently did not use a sufficiently large number of other individuals as sources of A serum to encounter one which did not contain Mo agglutinin. It is almost certain however, that the antibody in human A serum that he used, to study the differences in the agglutinogen content of mouse erythrocytes, was Mo agglutinin. At least, the agglutinin in human sera that he used was absorbed by mouse erythrocytes and it was shown in this work that mouse red cells absorb only Mo agglutinin and not α or β agglutinins or the rabbit cell agglutinin.

Summary and Conclusion. The performance of agglutination tests, using the blood cells of mice of 5 inbred strains and 8 types of F_1 and F_2 hybrid mice, have shown that human sera can be differentiated, apart from the usual intra-group reactions, by their ability to agglutinate mouse erythrocytes. The differentiation of human sera on this basis depended on the presence or absence of a specific agglutinin (Mo agglutinin). It was found to bear no specific relationship to either the A-B-O blood groups or the Rh factor. Five of the 37 human sera tested (approximately 14%) did not contain the Mo agglutinin. Mouse erythrocytes adsorbed the Mo agglutinin but not α and β agglutinins or the rabbit cell agglutinin. Human erythrocytes of the α and β group and rabbit red cells adsorbed their specific agglutinins, but not the Mo agglutinin.

16371

Rates of Digestion, Gastric Emptying and Intestinal Absorption of Starch.

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We have shown in previous studies[1,2,3] that the rates of gastric emptying and intestinal absorption of carbohydrates were closely related. Our work has also indicated, contrary to the findings of Cori,[4] that the rate of intestinal absorption of carbohydrates depended, among other things, upon the amount of administered carbohydrate. Much of our earlier work has been done with dextrose administered by forced feeding. This method has been called unphysiological by some workers, and it must be admitted that few animals habitually obtain their nourishment by stomach tube. On the other hand, we found it quite impossible to study dextrose absorption in short time intervals by using any method other than forced feeding. Starch, either in pure form, or as part of some food product, lends itself to studies with voluntary feeding. We have shown in an earlier study that rats can be made to consume limited amounts of such starch in a relatively short period of time.

The present study was undertaken to obtain further information on the fate of voluntarily ingested starch, to extend our previous experiments to other cereal products, and to determine the role salivary digestion plays in the utilization of starch by the rat.

Methods. This investigation was carried out with 3 preparations—(1) corn starch added to boiling water in amounts giving a concentration of 10%; (2) a breakfast cereal from which the free sugar was removed by washing with water, followed by drying at 70°C (Cereal I); and (3) a breakfast cereal which contained no free sugar (Cereal II). The two breakfast cereals were fed as a paste, water being added in sufficient amounts to give a starch concentration of 10%. All 3 preparations were analyzed for starch, protein, fat, fiber, ash and moisture. As experimental animals we used white rats weighing between 140 and 200 g. These animals were fed the 3 starch preparations in amounts containing 247 or 317 mg of starch. In all other respects the procedure was the same as described by Fenton and Pierce.[3]

An effort was made to determine the extent of digestion in the alimentary tract above the pylorus. Some information was obtained by carrying out the standard osazone tests on the gastric contents. Since the presence of dextrins gave rise to abnormal crystal shapes, solubility in hot and cold water and in glacial acetic acid was used as the chief criterion. Additional information was obtained from a combination of Barfoed's and Benedict's qualitative tests.

Results and Discussion. The qualitative tests performed on the gastric and intestinal contents support the conclusion that the free sugar present in greatest amounts in the stomach was maltose while dextrose was found in the intestines. We have, therefore, calculated our results on this basis.

All pertinent data have been assembled in Table I. In general the rates of hydrolysis, emptying and absorption were closely related. The only exception to this was noted in the group receiving corn strach at the low level (247 mg of starch) with an absorption period of one hour. With this one exception the rates of hydrolysis, emptying and absorption of Cereal II and of corn starch were of the

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1 Fenton, P. F., *Am. J. Physiol.*, 1945, **144**, 609.

2 Birchall, E. F., Fenton, P. F., and Pierce, H. B., *Am. J. Physiol.*, 1946, **146**, 610.

3 Fenton, P. F., and Pierce, H. B., *Am. J. Physiol.*, 1947, **148**, 296.

4 Cori, C. F., *J. Biol. Chem.*, 1925, **66**, 691.

TABLE I.
Summary of Results Obtained with Rats Fed 3 Starch Preparations Fed at 2 Levels.*

Amount of starch fed	247 mg		317 mg	
Duration of experiment	1 hr mg	2 hr mg	1 hr mg	2 hr mg
Hydrolyzed				
Cereal I	137 ± 7.3†	179 ± 7.7	158 ± 8.5	213 ± 8.3
Cereal II	184 ± 3.8	222 ± 3.1	231 ± 5.2	258 ± 5.8
Corn starch	180 ± 5.8	222 ± 4.3	254 ± 5.2	281 ± 4.8
Emptied				
Cereal I	118 ± 8.8	170 ± 8.3	140 ± 10.0	198 ± 10.4
Cereal II	154 ± 3.8	210 ± 4.6	176 ± 6.2	226 ± 7.2
Corn starch	116 ± 8.2	197 ± 7.6	168 ± 9.6	248 ± 8.3
Absorbed				
Cereal I	117 ± 8.8	170 ± 8.3	130 ± 9.4	195 ± 10.1
Cereal II	148 ± 4.4	210 ± 4.6	158 ± 6.2	220 ± 7.8
Corn starch	115 ± 8.0	197 ± 7.6	165 ± 9.8	248 ± 8.3

* The data presented here were obtained on a total of 240 animals, each group containing from 15 to 30 animals. All values are expressed in terms of starch.
† Standard error

same order of magnitude. Cereal I was hydrolyzed, emptied and absorbed at a consistently slower rate.

The data presented here are in excellent agreement with our previous findings. It is again evident that increasing the size of the meal fed also increases the amount of starch hydrolyzed, emptied and absorbed. The fact that the rates of absorption decreased with time should not be emphasized too strongly since only relatively small amounts of carbohydrate remained unabsorbed during the second hour.

The intimate relationship of the rates of hydrolysis, emptying and absorption and the effect of varying the size of the meal upon these rates support very well our earlier reports in connection with the utilization of dextrose. The results reported here were, however, obtained under conditions which permitted the elimination of forced feeding.

Conclusions. Three starch preparations were fed to a total of 240 animals. The rates of hydrolysis, emptying and absorption are closely related. Increasing the size of the test meal increases these rates. The data were obtained under conditions permitting the elimination of forced feeding.

16372

A Comparison of the Nutritive Value of Egg Proteins and Their Amino Acid Content.

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Since the early investigations of Osborne and Mendel[1] the high nutritive value of egg proteins has been recognized. Mitchell and Carman[2] found that whole egg protein fed at an 8% level in the diet had a biological value of 93 as compared with 74 for pork and 67 for wheat. Subsequently the same investigators[3] reported a biological value of 94 for the nitrogen of whole egg and 83 for the nitrogen of egg albumin. Sumner[4] and others reported the biological value of whole egg protein as 94 when fed at a 5% level and 85 when fed at an 8% level and that the biological value was higher for young rats than

for older rats. It was also stated that egg proteins are superior to milk proteins in maintaining nitrogen balance of adult human subjects. Murlin and others[5] gave 97 as the biological value of whole egg protein for human subjects as compared with values of 81, 84, and 83 for soy bean, beefsteak, and peanut proteins, respectively. Hoagland and Snider[6] found that the average growth-promoting value of the proteins in dehydrated pork was 3.75 g gain in body weight per gram of protein consumed in tests with young rats as compared with a gain of 4.28 g for the protein in spray dried eggs. More recently Hoagland, Ellis, Hankins, and Snider[7] stated that the superiority of egg to pork protein was due to the notably more cystine and methionine it contained. Supplementing pork protein with either cystine or methionine gave a growth promoting value equal to that of the protein in eggs.

Mitchell and Block[8] have taken the amino acids of whole egg protein as a standard for comparison of other proteins in an effort to assay biological values on the basis of essential amino acid content. Egg albumin is deficient in both cystine and methionine in comparison with whole egg protein, no data on whole egg white protein or on whole yolk protein were given. Calvery and Titus[9] have studied the sulfur, tryptophane, and cystine content of the whole white and whole yolk proteins and of egg albumin. The whole white protein was higher in all 3 substances than either the albumin or yolk proteins. The whole white protein contained 1.66% sulfur and the yolk protein contained 1.19%. Munk and others[10] found that the total egg protein was composed of 65% white protein and 35% yolk protein. On this basis the sulfur content of whole egg protein should be approximately 1.5%. Patton and Palmer[11] found the sulfur content of the whole egg protein to be 1.35%. Munk[10] found practically the same cystine content in the white as in the yolk protein, 1.9% and 2.2% respectively. Methionine was determined by subtracting the cystine sulfur from the total sulfur and considering the difference as due to methionine. On this basis the whole white protein contained 6.6% and the yolk protein 3.0% methionine. The tryptophane content of the 2 proteins was identical, 1.4%, the phenylalanine content of the whole white protein was 6.2%, approximately 50% greater than the phenylalanine content of the yolk protein. Differences in the other essential amino acids were slight. No feeding studies were reported.

We have prepared whole egg, whole white, and yolk proteins and analyzed them for nitrogen, sulfur, cystine, cysteine, methionine, phenylalanine, tryptophane, threonine, histidine, lysine, and arginine. The same proteins were used in feeding experiments with young rats to determine their protein efficiency values. In addition 3 commercial egg proteins were run similarly.

Experimental. Preparation of proteins. The egg whites and yolks were separated and poured into acetone. After stirring vigorously they were allowed to stand overnight when the acetone was decanted and replaced with fresh acetone. This acetone treatment was repeated 4 times. The whole white protein was prepared similarly. After the final treatment with acetone all the proteins were fairly granular. Following the acetone treatment

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, **15**, 311; 1916, **20**, 35.

[2] Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1924, **60**, 613.

[3] Mitchell, H. H., and Carman, G. G., *J. Biol. Chem.*, 1926, **68**, 183.

[4] Sumner, E. E., Pierce, H. B., and Murlin, J. R., *J. Nut.*, 1938, **16**, 37; Sumner, E. E., *J. Nut.*, 1938, **16**, 129; Sumner, E. E., and Murlin, J. R., *J. Nut.*, 1938, **16**, 141.

[5] Murlin, J. R., Edwards, L. E., and Hawley, E. E., *J. Biol. Chem.*, 1944, **156**, 785.

[6] Hoagland, R., and Snider, G. G., *Food Research*, 1946, **11**, 494.

[7] Hoagland, R., Ellis, N. R., Hankins, O. G., and Snider, G. G., *J. Nut.*, 1947, **34**, 43.

[8] Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, 1946, **163**, 599.

[9] Calvery, H. O., and Titus, H. W., *J. Biol. Chem.*, 1934, **105**, 683.

[10] Munks, B., Robinson, A., Beach, E. F., and Williams, H. H., *Poultry Sci.*, 1945, **24**, 549.

[11] Patton, A. R., and Palmer, L. S., *J. Nut.*, 1936, **11**, 129.

TABLE I.
Amino Acids in Egg Proteins.
All values, except nitrogen, are on the basis of the protein containing 16% nitrogen. Nitrogen values are expressed on the ash and moisture-free original protein.

	Whole egg				Commercial whole egg		
	I	II	Whole white	Whole yolk	I	II	Commercial whole white
Amino acid	%	%	%	%	%	%	%
1. Cystine	2.26	2.25	2.48	1.82	1.33	2.11	2.33
2. Cysteine	0.28	0.10	0.41	0.14	0.00	0.00	0.23
3. Methionine	3.95	3.88	4.15	3.61	4.30	3.65	3.96
Phenylalanine	4.46	4.50	5.42	3.73	4.84	4.87	5.29
Tryptophane	1.29	1.42	1.40	1.16	1.47	1.35	1.56
Sulfur—total	1.57	1.56	1.78	1.30	1.38	1.40	1.62
Sulfur calculated from 1 + 2 + 3	1.53	1.46	1.67	1.30	1.28	1.35	1.54
Nitrogen	13.76	12.62	14.53	12.78	13.34	12.67	12.64
Arginine	6.54	6.46	5.55	6.98	6.40	6.59	6.29
Histidine	1.53	1.43	1.20	1.50	1.38	1.61	1.29
Lysine	5.14	5.05	4.83	5.81	5.09	5.13	5.20
Threonine	3.78	3.88	4.10	3.60	3.94	3.82	4.42

the proteins were treated overnight with ethyl alcohol. The alcohol was removed by filtration and the residues extracted with ethyl ether by allowing them to stand overnight in contact with the ether. The ether extraction was repeated twice. The final products were air-dried.

The commercial proteins studied included a dried egg white and 2 dehydrated, defatted whole egg preparations, both made by the same manufacturer.

Analysis. Total nitrogen was determined on all samples by the macro Kjeldahl procedure. Total sulfur was determined by the method of Pollack and Partansky.[12] Phenylalanine, methionine, and tryptophane were determined, following alkaline hydrolysis, as described by Hess and Sullivan.[13] Cystine and cysteine were determined by the method of Sullivan, Hess, and Howard[14] as used by Hess and Sullivan.[15] Threonine was determined by the method of Shinn and Nicolet.[16] Arginine, histidine, and lysine were determined by the methods described by Block and Bolling.[17] The values given in Table I are expressed on the basis of the proteins containing 16% nitrogen since this method was used in determining the protein content of the diet. The nitrogen values in the table, however, are on the original sample corrected for moisture and ash.

Feeding experiments. Albino rats of the Sprague-Dawley strain were used. The diets were made up with the egg proteins to contribute 10% protein to the diet. The nitrogen of

TABLE II.
Composition of the Diet.

Constituent	%
U.S.P. No. 2 salt mixture	4
Wilson's 1:20 liver concentrate	1
Crisco	5
Corn oil	5
Protein—N × 6.25 to contribute	10
Cerelose and corn starch to make up	100%.

Synthetic vitamins were added in the following quantities per 100 g of diet: alpha tocopherol 4.0 mg, 2-methyl-1-4-napthoquinone 1.0 mg, thiamine hydrochloride 0.8 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.8 mg, niacin 4.0 mg, calcium pantothenate 4.4 mg, para-aminobenzoic acid 4.0 mg, choline chloride 200.0 mg, and inositol 21.6 mg. Vitamins A and D were fed separately, 3 drops of reference cod liver oil weekly.

In the diet containing 0.25% cystine an equal weight of corn starch was omitted.

[12] Pollack, R. N., and Partansky, R. M., *Ind. and Eng. Chem., Anal. Ed.*, 1934, **6**, 330.

[13] Hess, W. C., and Sullivan, M. X., *Ind. and Eng. Chem., Anal. Ed.*, 1945, **17**, 717.

[14] Sullivan, M. X., Hess, W. C., and Howard, H. W., *J. Biol. Chem.*, 1942, **145**, 621.

[15] Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, 1943, **151**, 625.

[16] Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, 1941, **138**, 91.

[17] Bosshardt, D. K., Ydse, L. C., Ayres, M. M., and Barnes, R. H., *J. Nut.*, 1946, **31**, 23.

TABLE III.
Growth of Weanling Rats on Egg Protein Diets.

Protein	No. of rats	Initial wt, g	Final wt, g	Gain, g	Food intake, g	PE ratio
Whole egg I	5	47.0*	97.0	50.0	151.4	3.30
		45.0	100.0	55.0	141.0	3.90
		55.0	108.0	53.0	177.0	2.99
Egg white	4	49.0	105.8	56.8	161.5	3.51
		54.0	120.0	66.0	177.0	3.73
		48.0	104.0	56.0	169.0	3.31
Yolk	4	46.8	96.0	48.8	151.0	3.23
		47.0	97.0	50.0	137.0	3.65
		49.0	106.0	57.0	191.0	2.98
Commercial egg white	8	49.8	104.1	54.4	161.1	3.37
		55.0	117.0	62.0	159.0	3.90
		45.0	87.0	42.0	168.0	2.50
Commercial whole egg I	9	49.4	90.9	41.4	148.4	2.80
		52.0	100.0	48.0	151.0	3.18
		44.0	83.0	39.0	159.0	2.45

* First value in each case is the average, followed by the values for the rats having the highest and lowest PE ratios in the group.

TABLE IV.
Growth of Weanling Rats on Egg Protein Diets.

Protein	No. of rats	Initial wt, g	Final wt, g	Gain, g	Food intake, g	PE ratio
Whole egg II	9	49.6*	82.1	32.6	104.4	3.12
		61.0	107.0	46.0	141.0	3.26
		60.0	94.0	34.0	124.0	2.74
Commercial whole egg II	9	48.8	73.2	24.6	100.9	2.33
		45.0	74.0	29.0	101.0	2.90
		50.0	68.0	18.0	103.0	1.75
Commercial whole egg II + 0.25% cystine	9	50.2	75.8	25.6	107.2	2.38
		44.0	70.0	26.0	89.0	2.92
		48.0	77.0	29.0	131.0	2.21

* First value in each case is the average, followed by the values for the rats having the highest and lowest PE ratios in the group.

the sample times 6.25 gave the protein content and the amounts of food consumed by the rats were carefully determined and recorded. Two series of feeding experiments were run. The average food intake and weight gains for a period of 2 weeks of each group of rats are given in Tables III and IV. Bosshardt and others[18] found that the protein efficiency ratio* reached a maximum in approximately 10 days and state that this period is sufficiently long to permit reliable calculations. If the experiments are run for a long period of time the PR ratio decreases.

Discussion. The first series of experiments, Table III, indicated that the whole white protein has a higher PE ratio than either the whole egg or yolk proteins. The commercial whole white protein has a PE ratio that is only slightly less than that of the laboratory whole white protein while commercial whole egg protein 1 has a markedly lower PE ratio than the laboratory prepared sample. In comparing the amino acid content of the laboratory prepared whole egg protein with that of commercial sample 1 it was noted that the sulfur amino acid content of the commercial sample was markedly lower. No

* The protein efficiency ratio (PE ratio) is calculated by dividing the weight gained by the grams of protein ingested.

marked differences were noted in the content of the other essential amino acids.

In an effort to determine whether the variation in total sulfur amino acid content, particularly cystine, would explain the difference in the PE ratio another series of feeding experiments were run. A new preparation of whole egg protein and another sample of commercial whole egg protein from the same producer were fed at the same level as previously. At the same time 0.25% cystine was added to the commercial whole egg protein diet and fed to another set of rats. These results are given in Table IV. The PE ratio of whole egg protein II was 3.13 as compared with the 3.3 in the first series while commercial whole egg protein II was 2.33 compared with 2.8 in the first series. The commercial whole egg protein II plus cystine had a PE ratio of 2.38, practically identical with that of the diet without the added cystine. It will be noted from Table I that whole egg protein II has an amino acid content the same as that of Sample I within the limits of experimental error. Commercial whole egg protein II contains slightly more cystine than commercial Sample I and slightly less methionine, there is little variation in all the samples of whole egg proteins in the other essential amino acids tested. It is apparent that the addition of cystine has not improved the PE ratio of the commercial whole egg protein. The only essential amino acids which were not determined in the proteins are leucine, isoleucine, and valine. These, according to Mitchell and Block,[8] are present in such large amounts in whole egg protein that it is difficult to imagine any lack of these amino acids in any of the samples. These results emphasize the difficulty of assaying the nutritional values of proteins by analyzing for the essential amino acids. In spite of the similarity of the amino acid content of the commercial whole egg and laboratory whole egg proteins, except for cystine, they are biologically different, even when the commercial sample is reenforced with cystine.

Summary. The proteins of whole egg, whole egg white, and yolk, were prepared and analyzed for cystine, methionine, phenylalanine, tryptophane, threonine, arginine, histidine, lysine, sulfur, and nitrogen. These proteins were incorporated into diets at a 10% level and fed to young rats. The protein efficiency ratio (gain in weight per gram of protein ingested) was determined after a 2-week feeding period. Commercial preparations of whole egg and whole white protein were similarly analyzed and their PE ratio determined. The PE ratio of the whole white protein was higher than that of either the whole egg or the yolk proteins. The PE ratio of the commercial whole white protein was almost as high as that of the laboratory prepared sample. The PE ratio of commercial whole egg protein was markedly lower than that of the laboratory prepared sample. The amino acid content of all the whole egg proteins were quite similar with the exception of the cystine content of the commercial samples which was lower than that of the laboratory samples. When the commercial whole egg protein II was reenforced with 0.25% cystine the PE ratio was not improved. Other factors in addition to the amino acid content apparently play a role in the biological value of proteins.

16373 P

Effect of Potassium on the Ventricular Deflections of the Electrocardiogram in Hypertensive Cardiovascular Disease.*

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Many reports have been published concerning the effects of potassium on the electrocardiogram in various clinical states[1–13] and in experimental animals.[14–17] These have been concerned chiefly with its effect upon the T-wave. Relatively little attention has been devoted to its effect upon the QRS complex except in those cases in which toxic doses were given. Inspection of the published records, however, shows that in many instances a reduction in the size of the QRS deflections followed the administration of this drug. We refer particularly to instances in which potassium was given to patients with hypertensive heart disease. In the articles referred to the QRS changes were not mentioned in the texts or legends. In fact in some of these it was reported that no change in the form of these deflections took place.[4,13] In a previously reported study[18] sodium restriction in hypertensive vascular disease was followed by retrogressive electrocardiographic changes unaccompanied by a significant change in blood pressure. This raised the question as to whether a relative increase in tissue potassium secondary to sodium restriction was responsible for these electrocardiographic phenomena. Consequently the effect of potassium alone upon the electrocardiogram in cases of hypertension was investigated. Potassium in the form of the chloride, dibasic phosphate, or citrate was administered, on 25 occasions, to patients with abnormal electrocardiogams characteristic of hypertensive vascular disease, and, on ten occasions, to normal subjects. Electrocardiograms were obtained immediately before and at times ranging from 15 minutes to 3 hours after the ingestion of 5 to 20 g of one of the potassium salts mentioned. In several instances 10 to 24 g of potassium salts daily had been prescribed previously and the patient had continued on this dosage up to the time of the test.

In every instance in which potassium was

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1 Bamber, J. M., *Am. J. Med. Sci.*, 1935, **189**, 681.

2 DeWesselow, O. L. V. S., and Thomson, W. A. R., *Quart. J. Med.*, 1939, **8**, 361.

3 Thomson, W. A. R., *Lancet*, 1939, **1**, 808.

4 Thomson, W. A. R., *Brit. Heart J.*, 1939, **1**, 269.

5 Stewart, H. J., Smith, J. J., and Milhorat, A. I., *Am. J. Med. Sci.*, 1940, **199**, 789.

6 Stewart, H. J., and Smith, J. J., *Am. J. Med. Sci.*, 1941, **201**, 177.

7 Keith, N. M., Osterberg, A. E., and Burchell, H. B., *Ann. Int. Med.*, 1942, **16**, 879.

8 Keith, N. M., and Osterberg, A. E., *Proc. Staff Meet., Mayo Clinic*, 1946, **21**, 385.

9 Sampson, J. J., Alberton, E. C., and Kondo, B., *Am. Heart J.*, 1943, **26**, 164.

10 Sharpey-Schafer, E. P., *Brit. Heart J.*, 1943, **5**, 80.

11 Sharpey-Schafer, E. P., *Brit. Heart J.*, 1943, **5**, 85.

12 Keith, N. M., Burchell, H. B., and Baggenstoss, A. H., *Am. Heart J.*, 1944, **27**, 817.

13 del Castillo, E. B., and Onativia, E. A. y A., *Medicina*, 1945, **6**, 471.

14 McLean, F. C., Bay, E. B., and Hastings, A. B., *Am. J. Physiol.*, 1933, **105**, 72.

15 Winkler, A. W., Hoff, H. E., and Smith, P. K., *Am. J. Physiol.*, 1938, **124**, 478.

16 Winkler, A. W., Hoff, H. E., and Smith, P. K., *Am. J. Physiol.*, 1939, **127**, 430.

17 Chamberlain, F. L., Scudder, J., and Zwemer, R. L., *Am. Heart J.*, 1939, **18**, 458.

18 Bryant, J. M., and Blecha, E., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 227.

given to a patient with hypertensive heart disease accompanied by the characteristic changes in the electrocardiogram there was a reduction in voltage of the QRS deflections which persisted for several hours. Frequently there was also a reduction in the length of the QRS interval. In the vast majority of cases the T-wave became less inverted or upright when it was originally inverted, or taller when it was originally upright. Serial electrocardiographic observations showed that following the administration of potassium the electrocardiogram passes in reverse order through the same series of configurations that it passes through in the course of the development of the changes characteristic of hypertensive heart disease. In a few instances the T-wave became more deeply inverted after potassium even though the voltage of the QRS deflections was reduced. In the majority of instances the mean axis of the QRS shifted to the right of its original position while that of the T-wave was shifted to the left. The magnitude of the ventricular gradient was usually increased and its direction was shifted slightly to the right.

In normal subjects the administration of potassium was followed by electrocardiographic changes similar in character but less pronounced than those seen in cases of hypertension. In one patient with left ventricular enlargement due to luetic aortic insufficiency, but with no definite increase in blood pressure, the administration of potassium was followed by pronounced toxic symptoms and transient left bundle branch block.

In hypertensive vascular disease sodium restriction alone is followed by electrocardiographic changes identical with those produced by the administration of potassium. No close correlation between the changes in blood pressure and the changes in the electrocardiogram is apparent. It should be pointed out that the electrocardiographic changes following sympathectomy in hypertensive patients are of the same type, and, that in this case also these changes are not always very closely related to the effects on the blood pressure.

In calling attention to the similarity between the changes in the electrocardiogram produced by the administration of potassium in cases of hypertensive heart disease, and the changes in the electrocardiogram which often follow sympathectomy or the restriction of sodium in cases of the same kind, we do not imply that the mechanism by which these electrocardiographic changes is brought about is necessarily the same in both instances. This is a question which can be decided only by future investigations.

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A Gaseous Nitrogen Elimination Test to Determine Pulmonary Efficiency.

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Nearly all physiologists interested in respiration have contributed to our knowledge of nitrogen elimination produced by decompression or by oxygen inhalation. As space prevents an adequate review of the literature, we can only refer the reader to typical references of studies by Haldane and Priestley,[1]

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1 Haldane, J. S., and Priestley, J. G., *Respiration*, New Haven, Yale University Press, 1935, 493 pp.

2 Berggren, S. M., *Acta physiol. Scandinav.* 1942, **4** (Suppl. 11).

Berggren,[2] Behnke,[3] Bornstein,[4] Lundsgaard and Van Slyke,[5] Christie,[6] Cournand and his associates,[7] and Bateman.[8]

As a result of the ease and rapidity of analysis possible with the nitrogen meter devised by Lilly and Anderson[9] of the Johnson Foundation it is now possible to make, as frequently as desired, readings of the nitrogen content of oxygen-nitrogen mixtures. For the principle and details of this nitrogen analyzer the reader should consult the original paper. For our use Baldes enlarged the lower part of the scale (0 to 15%) so that differences in the nitrogen content of CO_2-free nitrogen-oxygen mixtures at constant temperature and saturated with water vapor can be determined with an accuracy of 0.05% points provided duplicate analyses in Haldane high oxygen gas-analyzers are made at the beginning and end of each experiment for the purpose of calibration; the intermediate readings are linear.

The respiratory apparatus used is a closed circuit system consisting of a spirometer, soda-lime container, filter and a blower with corrugated tube connections to the valve attached to mask or mouthpiece.

The spirometer records both respiratory movements and ventilation rate and has a fan on the floor for rapid mixing of gases. For an experiment it is filled with 40 to 60 liters of oxygen (99.6 $\pm$ 0.1% purity) so that at the end of the 30-minute experiment the nitrogen content does not exceed 8 to 10%. Wet and dry bulb thermometers are placed in the air stream just as the circulating mixture leaves the gasometer. A sampling tube leads to the nitrogen meter which draws out approximately 6 cc per minute. The blower produces a flow of 80 to 100 liters per minute through the closed circuit system. Therefore the over-all time lag is less than 0.2 minute from the moment the expired air enters the system to the meter reading of the nitrogen content of the mixed gases.

The experiment itself is conducted like a basal metabolism test except that it lasts 30 minutes. The valve to start the experiment must be turned exactly at the bottom of the subject's natural expiration to avoid a bothersome correction. Just before the experiment is ended the vital capacity is determined by 2 cycles of maximal inspiration and expiration; the spirometer tracing is then continued for 5 minutes to establish accurately the average expiratory level from which the different pulmonary subdivisions are calculated.

The accumulated nitrogen elimination (BTPS) is a simple calculation and should be made for 15 to 20 different points in the 30 minute experimental period. For plotting we have found the use of 2 by 3 cycle log-log paper very convenient, for the following reasons: 1. The time scale covers 3 log units (0.2 to 30 minutes). 2. As first shown by the experiments of Boothby, Lovelace and Benson,[10,11] the body tissue nitrogen, both when the subject is at rest and when he is at work, when accumulated and plotted on log-log paper forms straight lines for more than 200 minutes; the slope of the line is in the main dependent on the rate at which the venous blood returns to the lungs. 3. The accumulated nitrogen coming from the air in the lungs rises very rapidly from the beginning of the experiment; when the pulmonary nitrogen has been completely eliminated, a sharp angle or knee is made as the further

3 Behnke, A. R., *U. S. Naval Med. Bull.*, 1937, **35**, 219.

4 Bornstein, A., *Z. f. exp. Path. u. Therap.*, 1918, **20**, 495.

5 Lundsgaard, Christen, and Van Slyke, D. D., *J. Exp. Med.*, 1918, **27**, 65.

6 Christie, R. V., *J. Clin. Invest.*, 1932, **11**, 1099.

7 Cournand, Andre, Baldwin, Eleanor deF., Darling, R. C., and Richards, D. W., Jr., *J. Clin. Invest.*, 1941, **20**, 681.

8 Bateman, J. B., *Proc. Staff Meet., Mayo Clin.*, 1946, **21**, 112.

9 Lilly, J. C., and Anderson, T. F., National Research Council, Division of Medical Science, CAM Report No. 299, 1944.

10 Boothby, W. M., Lovelace, W. R., II, and Benson, O. O., *J. Aeronautical Sci.*, 1940, **7**, 524.

11 *Physiology of Flight; Human Factors in the Operation of Military Aircraft.* A compendium of lectures and demonstrations given to Army Air Force personnel. The Aero Medical Research Laboratory, Experimental Engineering Section, Materiel Center, Wright Field, Dayton, Ohio, 1940-1942, p. 27, Fig. 15.

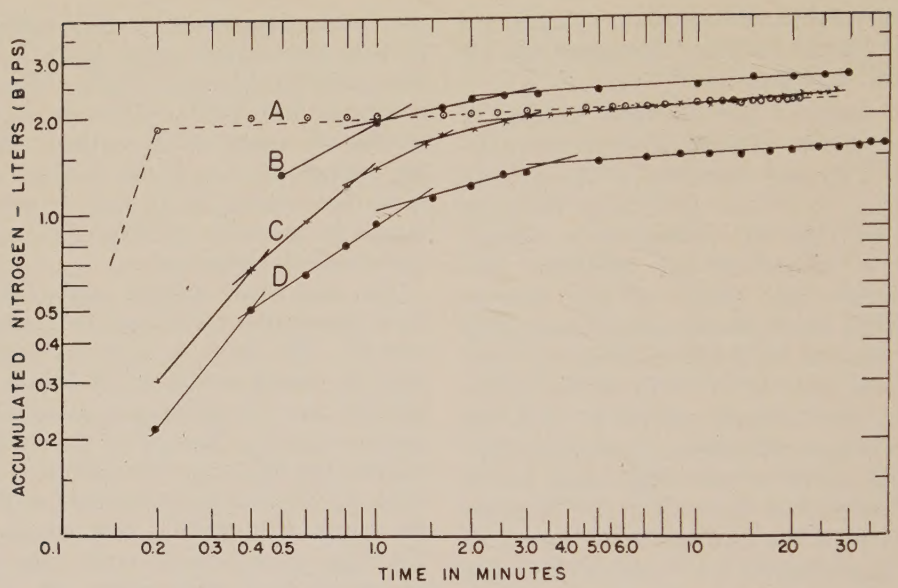

FIG. 1.

Characteristic curves plotted on log-log paper. Subject: man, aged 37 years; height 183 cm; weight 83.6 kg; surface area 2.07 square meters.

A. Subject started with 4 maximal respirations, then breathed normally.
B. The largest value for functional residual air volume in the series. Normal breathing.
C. The usual value for functional residual air volume in the series. Normal breathing.
D. The smallest value for functional residual air volume in the series. Normal breathing.

In B, C, and D the nitrogen washout time was between 2½ and 3½ minutes.
In A the washout time with 4 deep breaths was 0.2 minute.

accumulation of tissue nitrogen begins to form the straight line that continues for the duration of the experiment. The first part of the curve, representing essentially pulmonary nitrogen, may be in 2 or sometimes 3 phases which are important indicators of the degree and cause of respiratory inefficiency in some pulmonary diseases. 4. The final and characteristic bend or knee in the nitrogen elimination curve on log-log paper serves therefore as a convenient method of separating pulmonary from tissue nitrogen (Fig. 1). This graphic method permits one also to estimate, when desired, the comparatively small amount of tissue nitrogen that is eliminated along with the pulmonary nitrogen.

To calculate the functional residual air volume (BTPS) first read from the log-log plot the volume of nitrogen corresponding to the final bend or knee; second, divide this volume by the usual fraction of nitrogen normally present in alveolar air, 0.80, less the fraction of nitrogen in the inspired air at the time this knee is formed (which is usually about 0.05). The true residual air volume is obtained by subtracting the supplementary (reserve) air volume; the complementary and supplementary air volumes, the sum of which is the vital capacity, are obtained from the spirometer respiratory curve; the mean expiratory level is the base line from which the measurements are made. The total capacity is the vital capacity plus the true residual air volume. All air volumes are expressed at body temperature and pressure, saturated with water vapor (BTPS). They therefore represent the true size of the lung space available for respiration.

The oxygen consumption is determined as in any closed circuit system (after proper corrections) from the decrease in the spirometer volume during the experimental period.

The ventilation rate is also read from the spirometer tracing and the ratio between oxygen consumption and ventilation rate is calculated.

The efficiency of the subject's natural method of breathing is determined by the

FIG. 2.

Variations in the subdivisions of lung capacity as found in a series of 24 experiments on a normal subject at rest in a sitting posture. Subject: man, aged 37 years; height 183 cm; weight 83.6 kg; surface area 2.07 square meters. TC—total capacity; FR—functional residual air volume; CV—complementary air volume; SV—supplementary air volume; VC—vital capacity; RV—true residual air volume.

washout time required for elimination of the nitrogen that was in the pulmonary air; that is, the time after the subject begins breathing oxygen to the point on the plot where the knee or final bend occurs. In normal subjects under basal conditions this varies between 2 and 3.5 minutes; ventilatory inefficiency is increasingly severe as the washout time increases beyond 4 minutes; some patients with marked emphysema require 10 to 12 minutes to clear their lungs of pulmonary air nitrogen. In a normal subject under the same conditions the results are reproducible, as seen in Fig. 2.

Summary. An improved method for determining the nitrogen washout time with measurement of the various subdivisions of the pulmonary volume is described. The method is proving helpful in the differential diagnosis of pulmonary disease and in estimating the functional changes produced by intrathoracic operations.